Optimization of Triple-Helix-Directed DNA Cleavage by Benzoquinoquinoxaline – Ethylenediaminetetraacetic Acid Conjugates

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In memory of Claude Hélène.

The formation of triple-helical structures of DNA is based on sequence-specific recognition of oligopyrimidine·oligopurine stretches of double-helical DNA. Triple-helical structures can be stabilized by DNA-binding ligands. Benzoquinoquinoxaline (BQQ) derivatives are among the most potent intercalating-type agents known to stabilize DNA triple-helical structures. We previously reported the conversion of BQQ into a triplex-directed DNA cleaving agent, namely BQQ – ethylenediaminetetraacetic acid (EDTA), by coupling of 6-(3-aminopropylamino)BQQ to a suitable ethylenediaminetetraacetic acid derivative, and we demonstrated the ability of this conjugate to cause double-stranded cleavage of DNA at the triplex site. However, this prototype derivative BQQ – EDTA conjugate showed lower affinity towards triplex DNA than BQQ itself. In the light of this observation, and guided by molecular modeling studies, we synthesized a second generation of BQQ – EDTA conjugates based on 6-[bis(2-aminoethyl)amino]- and 6-(3,3′-diamino-N-methyldipropylamino) – BQQ derivatives. We confirmed by DNA melting experiments that the new conjugates displayed an increased specific affinity towards triple helices when compared to the previously synthesized BQQ – EDTA. In addition, the efficiency of these new agents in triplex-specific binding and cleavage was demonstrated by triplex-directed double-stranded cleavage of plasmid DNA.

KEYWORDS:

DNA cleavage · DNA-intercalating compounds · molecular modeling · oligonucleotides · triple helix

Introduction

Sequence-specific recognition of double-stranded DNA by an oligonucleotide can lead to the formation of a triple-helical structure.[1, 2] The triplex-forming oligonucleotide (TFO) binds to the major groove of the double helix and base triplets are then formed. Base composition of both the TFO and the oligopyrimidine·oligopurine target sequence dictate the configuration of hydrogen bonding (Hoogsteen or reverse Hoogsteen hydrogen bonds) and the orientation of the third strand oligonucleotide with respect to the oligopurine strand of the double helix.[3]

Considerable efforts have been devoted to increasing the thermal stability of DNA triple-helical structures under physiological conditions. For this purpose, two major strategies have been explored. One strategy involves chemical modification of one or more of the components in the third-strand oligonucleotide,[3, 4] and the other centers on the design of triplex-specific stabilizing ligands of DNA. Several different families of triplex-stabilizing ligands have been described. Certain ligands bind to the minor groove of double-helical DNA and others stabilize the triple-helical complex by intercalation between the base triplets.[5–8] One of the most effective triplex-stabilizing agents is the benzoquinoquinoxaline (BQQ) derivative **2** (Scheme 1). This compound discriminates efficiently between triplex and duplex DNA, with a much stronger affinity for triple helices.[9] We have

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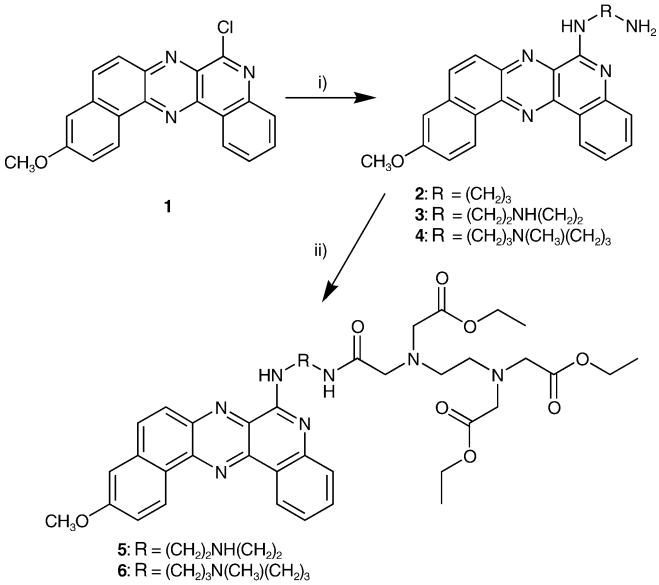
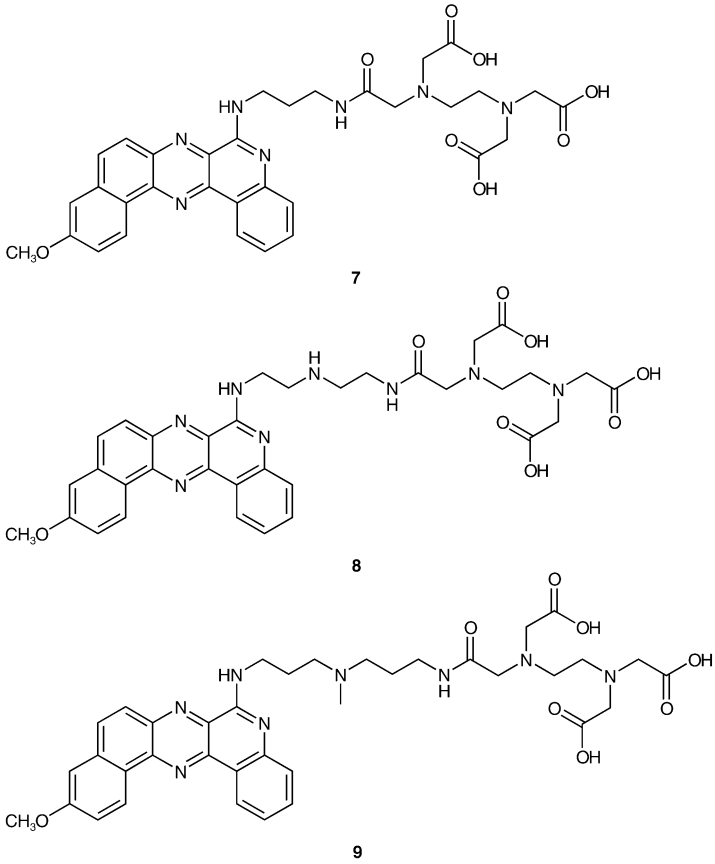


Scheme 1. Synthesis of BQQ – EDTA triethyl ester derivatives. i) Large excess of polyamine, 100 °C, 2 h. ii) EDTA – TEE (1 equiv), DCC (2.3 equiv), and HOBT (1.8 equiv) in DMF overnight. TEE, triethyl ester; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide.



Scheme 2. Chemical structures of the three different BQQ – EDTA derivatives.

previously reported the synthesis of a BQQ – ethylenediaminetetraacetic acid (EDTA) conjugate, and the use of this triplex-specific cleaving agent to provide evidence of the formation of different DNA triple-helical structures.[10]

We observed that the covalent attachment of BQQ **2** to ethylenediaminetetraacetic acid (EDTA) through its aminopropyl side chain resulted in a reduction of the triplex-stabilizing capacity of BQQ **2**. This effect was demonstrated by thermal denaturation experiments and DNA cleavage assays. As described herein, we synthesized two new BQQ – EDTA conjugates **8** and **9** (Scheme 2), which are optimally designed to retain a strong specific affinity towards DNA triple-helical structures. We have examined the triplex-specific stabilizing capacity of these new conjugates as well as their efficiency in triplex-directed cleavage of DNA at low concentrations.

Results and Discussion

In our initial effort to design a BQQ-based cleaving agent for triple-helical DNA, we chose to covalently link the intercalator by its 6-(3-aminopropylamino) side chain (as in **2**, Scheme 1), to ethylenediaminetetraacetic acid through the formation of an amide bond. However, although the derived BQQ – EDTA conjugate **7** (Scheme 2) is of fundamental interest, thermal denaturation studies showed that it is less potent at stabilizing triple-helical structures of DNA than the nonconjugated BQQ derivative **2** itself ($T_m = 45\,^\circ$C versus $69\,^\circ$C, respectively; see below). In addition, quantification of the extent of double-stranded cleavage of DNA triplex by this BQQ – EDTA conjugate **7** showed that a high concentration of the compound (50 μM) is required in order to attain 50 % cleavage. A large excess of the ligand led to undesired nonspecific cleavage outside the triple-helix site. We suspected that the reduced stabilizing efficiency of BQQ in its EDTA-conjugated form might have been caused partly by the loss of the positive charge of the aminoalkyl side chain. Indeed, whereas in BQQ **2** the free amino function is most likely protonated in the pH range used for the formation of a triple helix (pH 6 – 7), the amide bond in BQQ – EDTA **7** is not prone to protonation at these pH values. In addition, the relatively short length of the linking side chain in **7** could lead to a situation where the presence of the bulky EDTA component perturbs the ability of BQQ to bind and stabilize triple helices.

In the light of these results, we considered several chemical modifications of BQQ – EDTA that might restore the ability of the intercalator to efficiently stabilize triple-helical structures and direct double-stranded cleavage of DNA specifically to the triplex site. Thus, we began to examine the effect of a positively charged linker as well as that of a longer aliphatic chain on the stabilizing efficiency of BQQ. Accordingly, we synthesized two new BQQ – EDTA conjugates **8** and **9** (Scheme 2), which have different side chains. We were able to confirm by thermal denaturation experiments that these molecules exhibit increased specific affinity towards triple helices in comparison to the previous BQQ – EDTA **7**. These results align well with molecular modeling studies of the interaction of each of the

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three triplex-intercalating compounds with DNA. In addition, we showed the high efficiency of the new compounds in achieving triplex-specific double-stranded cleavage of plasmid DNA by using an in vitro cleavage assay in the presence of TFO.

Molecular modeling

Two aliphatic triamine linkers were considered in the design of the new BQQ – EDTA conjugates. As shown in Scheme 2, the aminoalkyl linkers in BQQ – EDTA conjugates **8** and **9** are longer than that present in compound **7**, and each of them contains an additional central amino group available for protonation.

These BQQ – EDTA conjugates were first manually docked into a triplex with the polyaromatic rings of BQQ intercalated between two base triplets, while the side chain bearing EDTA · Fe was located either in the minor or the major groove. An adiabatic mapping was performed to search for the minimal energy complex from several starting points. In all cases, a minimal conformational energy was found in the complex in which the EDTA · Fe moiety was located in the minor groove. The same calculations were performed for double-helical DNA structures and similar results were obtained. Table 1 shows the contributions made by various energy components to the energies of these complexes as well as their relative complexation energies (defined as the difference between triplex complexation energy and duplex complexation energy). It should be kept in mind that these energy calculations are purely indicative of trends, since ionic and hydration effects were not explicitly treated by molecular mechanics calculations in this work. However the results provide an important qualitative impression of the general energetic contribution of the systems.

Table 1. Analyses of the conformational energy-minimized models of the triple helix or the double helix complexed with BQQ or BQQ – EDTA conjugates.[a]

Ligands	DNA	LJ	Elec	ΔE_{DNA}	ΔE_{LIG}	E_C	ΔE_C
BQQ **2**	triplex	−55.2	−49.3	+30.0	+2.0	−72.5	−12.2
	duplex	−46.6	−44.3	+22.3	+8.4	−60.3	
BQQ – EDTA **7**	triplex	−66.8	−23.7	+29.0	+3.9	−57.6	−6.1
	duplex	−48.4	−23.9	+16.8	+4.0	−51.5	
BQQ – EDTA **8**	triplex	−70.3	−49.8	+27.7	+6.6	−85.8	−20.7
	duplex	−53.0	−45.9	+30.6	+3.2	−65.1	
BQQ – EDTA **9**	triplex	−75.2	−54.9	+38.1	+12.9	−79.1	−18.3
	duplex	−47.1	−49.6	+30.1	+5.8	−60.8	

[a] Complexation energy (E_C) is decomposed into Leonard-Jones (LJ) and electrostatic (Elec) interactions, as well as the deformation energies of nulceic acids and ligands (ΔE_{DNA} and ΔE_{LIG}). The relative complexation energy (ΔE_C) is calculated as the difference between the complexation energies for the triplex and that for the duplex (see the Materials and Methods section for details). Energies are given in kcal.

The relative complexation energies (ΔE_C) of all the complexes are negative, which indicates that triplex-specific binding occurs for all the BQQ – EDTA conjugates. As expected, the prototype conjugate, BQQ – EDTA **7** has a significantly reduced ΔE_C value compared to **2**, which can be ascribed to the reduction in electrostatic interaction. The use of a longer aliphatic triamine linker, which allows protonation at the central amine group and thereby creates a positive charge in the side chain, restored favorable electrostatic interactions and relative complexation energy. Figure 1 shows an energy-minimized model of BQQ – EDTA **8** complexed with a triple helix.

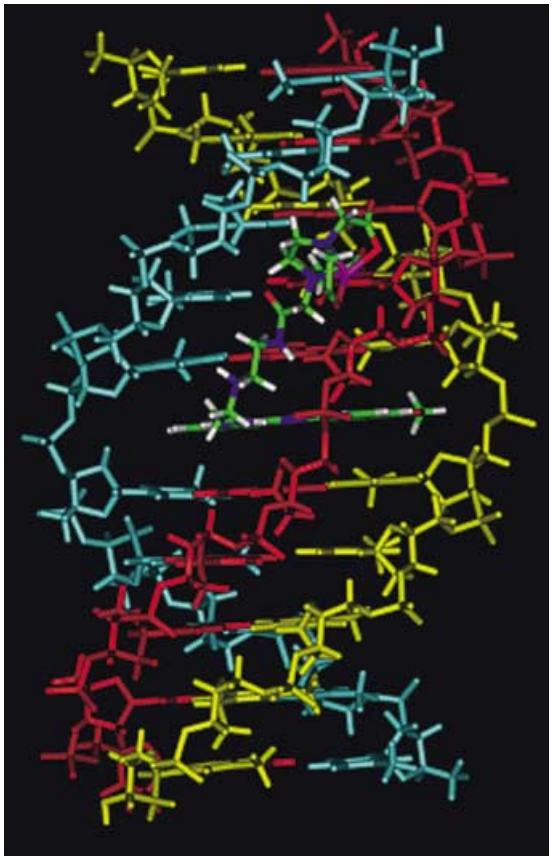

*Figure 1. Conformational energy-minimized complex of a triple helix with BQQ – EDTA **8**. The oligopyrimidine and oligopurine strands are shown in blue and red. The triple helix-forming oligonucleotide is in yellow. BQQ – EDTA **8** is in conventional atom colors and the linker and EDTA moiety are in the minor groove. Hydrogen atoms are omitted for clarity.*

Synthesis of BQQ – EDTA conjugates

Nonconjugated BQQ derivatives **3** and **4** (Scheme 1), which have *N*-(2-aminoethyl)ethane-1,2-diamine and *N*-(3-aminopropyl)-*N*-methylpropane-1,3-diamine side chains respectively, were obtained from **1** by using a standard protocol.[9] BQQ **3** and **4** were each coupled to ethylendiaminetetraacetic acid triethyl ester (EDTA-TEE) under DCC/HOBT conditions. The reaction of EDTA-TEE with the terminal amino group in BQQ derivatives **3** and **4** afforded the BQQ – EDTA triester intermediates **5** and **6** (Scheme 1) in 60 and 76% yields, respectively. The lower yield obtained for **5** was ascribed to the presence of a secondary amino group in the starting compound **3**. After purification by silica gel column chromatography, the triethyl esters **5** and **6** were hydrolyzed under alkaline conditions, and the derived

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triacid products were purified by reversed-phase column chromatography. The BQQ–EDTA conjugates **8** and **9** were thus obtained either as an ammonium trifluoroacetate salt of the triacid (**8**) or as a trilithium salt of the corresponding carboxylates (**9**; see the Materials and Methods section).

DNA melting experiments

It was observed that the BQQ–EDTA conjugates **8** and **9** themselves exhibited a reversible thermal transition associated with a pronounced hypochromicity in the absorbance of the BQQ moiety, with a red shift in wavelength (see the figures in the Supporting Information). These observations indicate that self-association of these BQQ–EDTA conjugates takes place. As the triplex melting experiments were performed with an excess of BQQ–EDTA (10 μM) over the DNA triple helices (1 μM), differential melting experiments were subsequently carried out as described below. The mixture of double helix and BQQ–EDTA was first prepared then separated into two samples. TFO or buffer (4.5 μL) was then added to these samples. Figure 2 shows the melting curves of triplex and duplex in the presence of BQQ–EDTA **7**, and

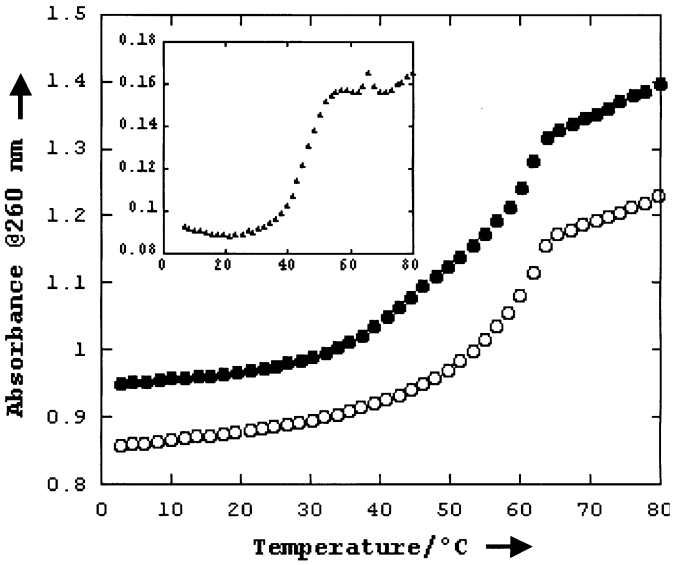

*Figure 2. Melting curves of triplex/BQQ–EDTA **7** (filled circles) and of duplex/BQQ–EDTA **7** (open circles). The differential melting curve is shown in the insert. DNA melting experiments were carried out in 10 mM sodium cacodylate buffer, 100 mM sodium chloride, pH 6.0.*

the differential melting curve of triplex + BQQ–EDTA **7**, where transitions ascribed to BQQ–EDTA **7** self-association and double helix ↔ single strands were subtracted. Only the transition triple helix ↔ double helix + third strand oligonucleotide was clearly observed in the differential melting curve. A differential melting curve was also recorded by placing the triplex + BQQ–EDTA **7** and duplex + BQQ–EDTA **7** samples in the sample and reference beams of the spectrophotometer, respectively. The same procedure was used for characterizing the triplex-stabilizing property of all the BQQ–EDTA conjugates. The melting temperatures of the triplex → duplex transition ($T_m^{3\rightarrow2}$) in the presence of the new BQQ–EDTA conjugates (**8** and **9**) were about 61 °C and 59 °C, respectively. Accordingly, these new conjugates stabilize the triple-helical structure of DNA by 42 °C and 40 °C, respectively, as compared to the $T_m^{3\rightarrow2}$ value of the triple helix itself. These compounds are, thus, considerably more efficient than BQQ–EDTA conjugate **7** at stabilizing the triple helix, as judged by the increased $T_m^{3\rightarrow2}$ values (+16 °C and +14 °C, respectively, Table 2).

***Table 2.** Melting temperatures of the triple-to-double helix transition in the presence of various BQQ derivatives.[a]*

Samples	$T_m^{3\rightarrow2}$ [°C] (±1)
Triple helix	19
Triple helix + BQQ **2**	69
Triple helix + BQQ–EDTA **7**	45
Triple helix + BQQ–EDTA **8**	61
Triple helix + BQQ–EDTA **9**	59

[a] DNA melting experiments were carried out in 10 mM sodium cacodylate buffer, 100 mM sodium chloride, at pH 6.0.

Triplex-directed cleavage of plasmid DNA

The plasmid (pTLX) used in this assay is a construct derived from a pUC12 vector plasmid in which a 27-bp oligopyrimidine·oligopurine sequence has been cloned.[11] This plasmid was first digested by XmnI, a unique-site restriction enzyme, and purified by precipitation. The linearized plasmid was then used to quantify the extent of triplex-directed cleavage of the new BQQ–EDTA conjugates. The resulting linear fragment of DNA formed a triple-helical structure upon incubation with a third-strand oligonucleotide complementary to the 27-bp oligopyrimidine·oligopurine sequence (10 mM sodium cacodylate buffer, 50 mM NaCl, pH 7.0). BQQ–EDTA (**7**, **8**, or **9**) was added, together with Fe^{2+} and the cleavage reaction was initiated by the addition of 2 mM dithiothreitol (DTT). As reported previously, this kind of triple-helix-directed cleavage of DNA would occur in the triplex site if the cleaving agent bound highly specifically to this structure, and the cleavage would consequently produce two DNA fragments of defined lengths. In our case, the size of these fragments would be 1901 and 817 bp if one reasons the cleavage would occur approximately in the middle of the 27-bp triplex site (Figure 3). For the three conjugates **7**, **8**, and **9**, the cleavage reactions were conducted in parallel to quantify and compare the yields of all three reactions in the presence of a triplex-forming third-strand oligonucleotide (TFO). Cleavage at both pH 6.0 and pH 7.0 were examined. It was found, in agreement with the results of others,[12] that EDTA·Fe compounds were less efficient at cleaving DNA at pH values lower than 7.0. Cleavage reactions were performed at either 4 or 37 °C. Under all examined conditions, quantitative gel analysis showed that the BQQ–EDTAs **8** and **9** had greater potency with regard to triplex-directed cleavage of DNA compared to the conjugate **7**. When a final concentration of 5 μM BQQ–EDTA conjugate **8** or **9**

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was chelated with 10 μM Fe^{2+} at 4 °C and pH 7, the yields of double-stranded cleavage at the site of formation of a triple helix were 66% and 72%, respectively (Figure 3). Under the same experimental conditions, conjugate **7** showed only 34% cleavage. In addition, it was possible to achieve reasonably good cleavage with a concentration of 1 μM BQQ–EDTA conjugate for

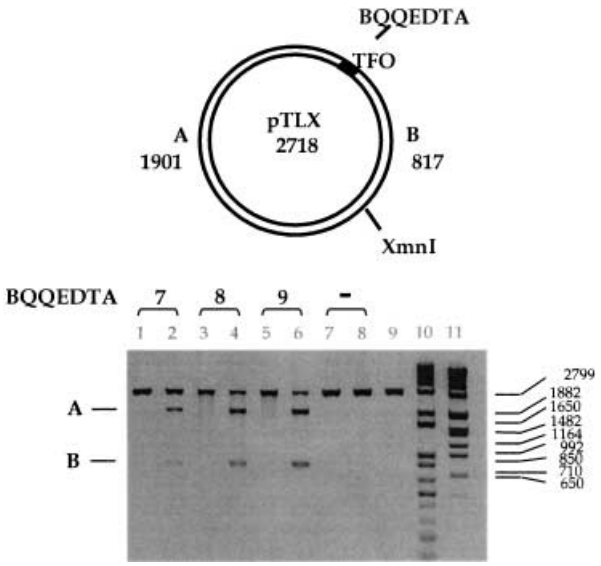

*Figure 3. Triplex-directed cleavage of a 2718-bp DNA fragment in the presence of 5 μM BQQ–EDTA derivative **7** (Lanes 1 and 2), **8** (Lanes 3 and 4), or **9** (Lanes 5 and 6), and a specific triplex-forming (Lanes 2, 4, 6, and 8) or a nonspecific oligonucleotide (Lanes 1, 3, 5, and 7) (20 μM), $Fe(NH_4)_2(SO_4)_2$ (10 μM), and DTT (5 mM) (see the Materials and Methods section for details). Lanes 7 and 8 show controls not treated with any BQQ–EDTA conjugate while Lanes 9, 10, and 11 are linear pTLX and molecular size markers, respectively.*

8 and **9** at pH 7.0 and 37 °C (54% and 60%, respectively) as compared to that achieved with BQQ–EDTA **7** (30%; data not shown). Cleavage reactions were analyzed after 18–22 h and no further attempt was made to drive the reactions to completion. In the presence of a control non-triplex-forming oligonucleotide, no notable cleavage of DNA was observed, which confirmed the triplex-specific character of these DNA cleavage reactions.

Previous work showed that the chemical attachment of a triplex-specific DNA ligand (BQQ **2**) to an EDTA·Fe conjugate leads to the formation of a new class of triplex-specific binding and cleaving compounds.[10] The weakness of Compound **7** lies in the fact that it does not retain the high triplex-stabilizing capacity of the parent compound, BQQ **2**, because of the noncharged aminopropyl side chain linking the BQQ to the EDTA component. In the work described herein, rational design of the linker by molecular modeling followed by experimental verification clearly demonstrate that the new longer and positively charged linkers are able to restore the triplex-stabilizing efficiency of the triplex-specific intercalator to the new BQQ–EDTA conjugates. The higher T_m values of triplex DNA in the presence of Compound **8** or **9** (Table 2) were consistent with the modeling study, which predicted an improved interaction between triple-helical structures and these new BQQ–EDTA conjugates (Table 1) as compared to that seen with **7**. Biochemical assays also confirmed that the efficiency of triple-helix-directed DNA cleavage by the newly synthesized BQQ–EDTA conjugates (**8** and **9**) is significantly improved. These new molecules can thus be used in micromolar concentrations either as artificial endonucleases for specific cleavage of double-stranded DNA fragments at triplex-forming sites, or as chemical probes to prove the formation of inter- as well as intramolecular triple helical structures, such as H-DNA.[13, 14]

Materials and Methods

Oligonucleotides (OligoGold grade) were synthesized and purified by Eurogentec (Seraing, Belgium) or by Genosys. The length of the oligonucleotides was controlled by electrophoresis. The syntheses of 6-chloro-11-methoxy-benzo[*f*]quino[3,4-*b*] quinoxaline (**1**) and BQQ–EDTA **7** were described elsewhere.[10] All other chemical reagents, including dithiothreitol (DTT), were purchased from Sigma or Aldrich. DNA markers were Roche Molecular Biochemicals products. BQQ–EDTA conjugates were dissolved in dimethylsulfoxide (DMSO).

Synthesis of *N*-(2-aminoethyl)-*N*′-(11-methoxybenzo[*f*]quino[3,4-*b*]quinoxalin-6-yl)ethane-1,2-diamine (BQQ 3) and *N*-(11-methoxybenzo[*f*]quino[3,4-*b*]quinoxalin)-6-yl-*N*′-(3-aminopropyl)-*N*′-methylpropane-1,3-diamine (BQQ 4): In separate experiments, Compound **1** (0.25 g) was heated for 2 h at 100 °C in the presence of a large excess (8 mL) of *di*(2-aminoethyl)amine or 3,3′-diamino-*N*-methyldipropylamine, respectively. The excess amine was then evaporated under reduced pressure. Water (30 mL) followed by 28% aqueous ammonia (2 mL) was added to the residue. BQQ **3** was isolated by filtration and recrystallized from toluene (70% yield). In the case of BQQ **4**, the resultant mixture was extracted from dichloromethane and the combined organic phases were dried with $MgSO_4$ and concentrated. The obtained BQQ **4** was converted into its maleate salt and recrystallized from ethanol (yield, 80%).

BQQ **3**: ^{1}H NMR ($CDCl_3$), $\delta = 9.32-7.14$ (9 H, Ar-H), 3.90 (s, 3 H, OCH_3), 3.81 (m, 2 H, CH_2-α), 2.99 (t, 2 H, CH_2-β), 2.73 (m, 4 H, CH_2-χ + CH_2-δ) ppm; FAB MS: calcd. for $C_{24}H_{25}N_6O$: 413.2090 $[M+H]^+$; found: 413.2079. BQQ **4**: ^{1}H NMR (DMSO-d_6), $\delta = 9.38-7.38$ (10 H, Ar-H), 6.08 (s, 6 H, CH=CH-maleate), 3.97 (s, 3 H, OCH_3), 3.75 (m, 2 H, CH_2-α), 3.18 (m, 4 H, CH_2-β + CH_2-χ), 2.86 (m, 2 H, CH_2-δ), 2.79 (s, 3 H, CH_3-N), 2.13, 1.9 (2 × m, 2 × 2 H, CH_2-ε + CH_2-ϕ) ppm; FAB MS: calcd. for $C_{27}H_{31}N_6O$: 455.2559 $[M+H]^+$; found: 455.2558.

Synthesis of BQQ–EDTA triethyl esters 5 and 6: DCC (2.3 equiv) and HOBT (1.8 equiv) were added to a solution of EDTA-TEE (0.10 g) in dimethylformamide (7 mL) and the mixture was stirred for 30 min at room temperature under an N_2 atmosphere. BQQ **3** or **4** (free base, 1.1 equiv) was then added and the mixture was stirred overnight at room temperature. The reaction was stopped by the addition of saturated aqueous sodium bicarbonate and the crude product was extracted with dichloromethane. The combined organic layers were washed with diluted aqueous sodium bicarbonate, dried with $MgSO_4$, and concentrated. The BQQ–EDTA triethyl ester conjugates **5** and **6** were each purified by neutral alumina (7% water) column chromatography (2% EtOH in dichloromethane for **5**; yield, 60%; pure dichloromethane for **6**; yield, 76%). BQQ–EDTA triethyl ester **5**: ^{1}H NMR ($CDCl_3$), $\delta = 9.33$ (d, 1 H, Ar-H, $J = 9.1$ Hz), 9.02 (d, 1 H, Ar-H, $J = 7.8$ Hz), 8.21 (t, 1 H, CO-NH), 7.91 (q, 2 H, Ar-H, $J = 3.6$ Hz), 7.67 (m, 2 H, Ar-H), 7.39 (t, 2 H, Ar-H, $J = 7.3$ Hz), 7.20 (m, 1 H, Ar-H), 4.09 (q, 6 H, CH_2-ethyl, $J = 7.0$ Hz), 3.98 (s, 3 H, OCH_3), 3.90 (m, 2 H, CH_2-α), 3.49 (s, 4 H, CH_2-CO), 3.46 (m, 2 H, CH_2-δ), 3.35 (s, 2 H, CH_2-CO), 3.29 (s, 2 H,

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CH_2-CO), 3.10 (t, 2H, CH_2-β, $J=5.5$ Hz), 2.91 (t, 2H, CH_2-χ, $J=5.7$ Hz), 2.75 (s, 4H, N-CH_2-CH_2-N), 1.18 (t, 6H, CH_3-ethyl, $J=7.1$ Hz), 1.17 (t, 3H, CH_3-ethyl, $J=7.0$ Hz) ppm. BQQ–EDTA triethyl ester **6**: ^{1}H NMR ($CDCl_3$), $\delta=9.25$ (d, 1H, Ar-H, $J=8.97$), 8.95 (dd, 1H, Ar-H, $J=7.9$, 1.3 Hz), 8.05 (t, 1H, CO-NH, $J=6.0$ Hz), 7.82 (d, 1H, Ar-H, $J=1.9$ Hz), 7.65 (m, 3H, Ar-H), 7.33 (m, 2H, Ar-H), 7.12 (d, 1H, Ar-H, $J=2.5$ Hz), 4.06 (q, 6H, CH_2-ethyl, $J=7.1$ Hz), 3.93 (s, 3H, OCH_3), 3.82 (m, 2H, CH_2-α), 3.44 (s, 4H, CH_2-CO), 3.31 (m, 4H, CH_2-CO + CH_2-ϕ), 3.24 (s, 2H, CH_2-CO), 2.73 (s, 4H, N-CH_2-CH_2-N), 2.62 (t, 2H, CH_2-χ, $J=6.7$ Hz), 2.51 (t, 2H, CH_2-δ, $J=7.3$ Hz), 2.34 (s, 3H, N-CH_3), 1.99 (m, 2H, CH_2-β, $J=6.6$ Hz), 1.81 (m, 2H, CH_2-ε, $J=7.3$ Hz); 1.17 (t, 9H, CH_2-ethyl, $J=7.1$ Hz) ppm.

Synthesis of BQQ–EDTA conjugates 8 and 9: A solution of triethyl ester **5** (0.11 g) in EtOH (5 mL) was treated with LiOH (6 equiv, 0.2 M) in water/ethanol (4:1, 10 mL) overnight at 4 °C. Purification of compound **8** was achieved by reversed-phase (25 × 170 mm Partisil 10 OD53 column; Whatman) high-pressure liquid chromatography (20–80% gradient of 70% aqueous acetonitrile/0.1% trifluoroacetic acid; 100 min; 40% yield). BQQ–EDTA **8**: ^{1}H NMR ($CDCl_3$), $\delta=8.60$ (m, 2H, Ar-H), 7.57 (m, 4H, Ar-H), 7.27 (m, 1H, Ar-H), 7.02 (m, 1H, Ar-H), 6.82 (m, 1H, Ar-H), 3.82 (m, 5H, OCH_3 + CH_2-α), 3.43 (t, 2H, CH_2-δ), 3.21 (s, 2H, CH_2-CO), 3.16 (s, 2H, CH_2-CO), 3.11 (s, 4H, CH_2-CO), 3.04 (m, 2H, CH_2-β), 2.88 (t, 2H, CH_2-χ), 2.61 (m, 4H, N-CH_2-CH_2-N) ppm. MALDI-TOF MS: calcd. for $C_{34}H_{39}N_8O_8$: 687.29 [M+H]; found: 687.22.

In a similar experiment, a solution of triethyl ester **6** (0.11 g) in MeOH (5 mL) was treated with LiOH (6 equiv, 0.2 M) in water/MeOH (4:1, 10 mL) overnight at 4 °C. Compound **9** was purified by silanized silica gel column chromatography (gradient, 0–20% MeOH in water; 78% yield). BQQ–EDTA **9**: ^{1}H NMR ($CDCl_3$), $\delta=8.59$ (m, 2H, Ar-H), 7.57 (m, 4H, Ar-H), 7.28 (m, 1H, Ar-H), 7.03 (dd, 1H, Ar-H), 6.85 (d, 1H, Ar-H), 3.82 (s, 3H, OCH_3), 3.79 (m, 2H, CH_2-α), 3.34 (m, 4H, CH_2-CO + CH_2-ϕ), 3.30 (m, 10H, 3 × CH_2-CO + N-CH_2-CH_2-N), 3.15 (m, 2H, CH_2-χ), 3.02 (m, 2H, CH_2-δ), 2.89 (s, 3H, N-CH_3), 2.26 (m, 2H, CH_2-β), 2.0 (m, 2H, CH_2-ε) ppm. MALDI-TOF MS: calcd. for $C_{37}H_{45}N_8O_8$: 729.34 [M+H]; found: 729.17.

Molecular modeling by energy minimization: The molecular mechanics calculations were carried out by using the JUMNA software (version 10) and Nchem program package.[15] Neither water nor positively charged counterions were explicitly included in the energy minimization. However, their effects were simulated by a sigmoidal, distance-dependent, dielectric function,[16] and by assigning half a negative charge to each phosphate group. Computations were carried out on a Silicon Graphics Indigo2 workstation. The EDTA·$Fe^{2+/3+}$ moiety was constructed with the InsightII program (Accelerys). As our aim was to compare the complexation energy of BQQ–EDTA conjugates which differ in the nature of the linker between the BQQ and the EDTA·$Fe^{2+/3+}$ moieties, an approximation was made to neglect the changes in the metalloorganic EDTA·$Fe^{2+/3+}$ complex upon complexation: we used a fixed moiety without net charge.

The coordinates of triple helices were derived from the previously published B-like triple helix stucture,[17, 18] which is now widely supported by many NMR and vibration spectroscopic studies. Typically, the triple helices were 10 base triplets in length. The two last base triplets at both ends were restrained to a mononucleotide symmetry in order to decrease end effects and to focus on the binding of BQQ–EDTA at the centre of the triplex, where an intercalation site was created by doubling the rise to 6.8 Å and subsequently reducing the twist by 20°. The BQQ–EDTA·Fe conjugates were first manually docked into the triplex with the polyaromatic plane of BQQ intercalated between two central base triplets, while the side chain bearing EDTA·Fe was located either in the minor or major groove. Care was taken to avoid strong steric clashes between EDTA·Fe and the triple helix. The same protocols were used for BQQ–EDTA·Fe intercalated into a canonical B-like double helix.

The complexation energy (E_C) was calculated by the addition of the Leonard–Jones (LJ) and electrostatic (Elec) interaction energies between the BQQ–EDTA·Fe conjugate and the nucleic acids. The deformation energy of the nucleic acids (double or triple helix) was evaluated approximately by finding the difference in conformation energy between complexed and uncomplexed nucleic acids (ΔE_{DNA}); the deformation energy of BQQ–EDTA·Fe (ΔE_{LIG}) was calculated in a similar way. Finally, the relative complexation energies (ΔE_C), calculated as the difference between the complexation energy with triplex and that with duplex DNA, were used to assess the specific binding of BQQ–EDTA·Fe to the triple helix.

Thermal denaturation experiments: Triple-helix stability was measured by using a dual-beam UV/Vis spectrophotometer. All thermal denaturation studies were carried out on a Uvikon 940 spectrophotometer interfaced with an IBM-AT personal computer for data collection and analysis. Temperature control of the cell holder was achieved by a Haake D8 circulating water bath. The temperature of the water bath was decreased from 80 to 0 °C and then increased again to 80 °C at a rate of 0.1 °C min^{-1} with the Haake PG 20 thermoprogrammer. The absorbance at 260 nm was recorded every 10 minutes (every 1 °C). Melting points were measured on a 36-bp duplex [5′-d(CTATCAGCTCAATCTTTTTTCTTCTTAACTCGTATC)-3′/3′-d(GATAGTCGAGTTAGAAAAAAGAAGAATTGAGCATAG)-5′] in the presence of a third strand oligonucleotide with the sequence 5′-d(TTCTTCTTTTTTCT)-3′ and a BQQ–EDTA conjugate (**7**, **8**, or **9**). This system represents approximately half the oligopyrimidine·oligopurine sequence used in the cleavage assay.

Cleavage assay on plasmid DNA: A 2718-bp linear fragment of DNA was obtained upon cleavage of pTLX[11] with a unique-site restriction enzyme, XmnI, followed by repeated precipitations (0.3 M NaOAc and ethanol). The DNA fragment was incubated (overnight at 4 °C) in sodium cacodylate buffer (10 mM), pH 6 or 7, containing NaCl (50 mM), and in the presence of a specific triplex-forming or a nonspecific third-strand oligonucleotide (20 μM). The sequences of the specific and nonspecific oligonucleotides were 5′-d(TTCTTCTTTTTTCTT-CTTCTTTTTTCT)-3′ and 5′-d(TCTCTCTCTCTCTCTCTCTCTCTCT)-3′, respectively. BQQ–EDTA **7**, **8**, or **9** (1–5 μM) was added to the above-mentioned samples and the reactions were left for 30 min at room temperature. $Fe(NH_4)_2(SO_4)_2$ (10 μM, 5 min) was added to each sample and the cleavage mixtures were initiated by the addition of DTT (5 mM). Cleavage reactions were left overnight at either 4° or 37 °C. Reaction mixtures were analyzed by 1% agarose-gel electrophoresis in the presence of ethidium bromide and the products were quantified by using ImageQuant software.

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