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Recognition and Cleavage of DNA by Rebeccamycin- or Benzopyridoquinoxaline Conjugated of Triple Helix-Forming Oligonucleotides

Paola B. Arimondo,[a] Pascale Moreau,[b] Alexandre Boutorine,[a] Christian Bailly,[c] Michelle Prudhomme,[b] Jian-Sheng Sun,[a,*] Thérèse Garestier[a] and Claude Hélène[a]

[a]*Laboratoire de Biophysique, UMR 8646 CNRS-Muséum National d'Histoire Naturelle, INSERM U201, 43 rue Cuvier, 75231 Paris, Cedex 05, France*

[b]*Laboratoire de Chimie Organique Biologique, Université Blaise Pascal, CNRS URA 485, 63177 Aubière, France*

[c]*INSERM U524 and Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Place Verdun, 59045 Lille, France*

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Abstract—Indolocarbazole and benzopyridoquinoxaline derivatives have been shown to have anti-tumor activity and to stimulate DNA topoisomerase I-mediated cleavage. Two indolocarbazole compounds (R-6 and R-95) and one benzopyridoquinoxaline derivative (BPQ(1256)) were covalently attached to the 3′-end of a 16mer triple helix-forming oligonucleotide (TFO). These conjugates bind to DNA with a higher affinity than the unsubstituted oligonucleotides. Furthermore, they induce topoisomerase I-mediated and triplex-directed DNA cleavage in a sequence-specific manner.

Introduction

Sequence-specific modulation of gene expression can be achieved by oligonucleotides which bind either to messenger RNAs in the 'antisense' strategy (leading to translation inhibition) or to double-helical DNA via triplex formation in the 'antigene' strategy (leading to transcription inhibition).[1]

At present, there are several limitations, other than the necessity of an oligopyrimidine•oligopurine target sequence, to the development of the antigene strategy. Except for a few cases,[2–4] the stability of triple-helical complexes is usually weak under physiological conditions, due, in part, to the electrostatic repulsion between the third strand and the duplex, as well as the requirement for the cytosines on the third strand to be protonated in the pyrimidine motif. A wide variety of DNA intercalators, benzopyridoindoles (BPI) and benzopyridoquinoxalines (BPQ), benzoquinoquinoxalines (BQQ), benzoindoloquinoline (BIQ), coralyne, anthraquinones, disubstituted-anthracene, dibenzophenantrolines,[5] have been shown to stabilize triple-helical structures. Covalent attachment of an intercalator[1,6–9] or a polyamine[10] to an oligonucleotide provides an additional binding energy that stabilizes the complex.

In this study, we have covalently attached an indolocarbazole derivative (R-6 or R-95) or a benzopyridoquinoxaline derivative (BPQ(1256)) to the 3′-end of a triple helix-forming oligonucleotide (TFO). Rebeccamycin derivatives (indolocarbazole family) have been chosen for their DNA intercalation properties.[11] They have also been identified as efficient anti-tumor drugs,[12–15] which induce topoisomerase I-mediated cleavage. Topoisomerase I is an ubiquitous nuclear enzyme, which alters DNA topology by transient DNA cleavage through an intermediate phosphodiester–tyrosine linkage. Several antineoplastic drugs, such as the camptothecin and the rebeccamycin analogues, have been shown to stabilize this transient intermediate complex[16,17] and are defined as Topo I poisons. The benzopyridoquinoxaline BPQ(1256) is a specific triple-helix ligand[7,8] and has been shown to stabilize triplex

Abbreviations: TFO, triple helix-forming olignucleotide; R-, indolocarbazole derivatives; BPQ, benzopyridoquinoxaline; Topo I, topoisomerase I; CPT, camptothecine; base triplets are noted as Y•R∗Z, where • indicates Watson–Crick hydrogen bonding and ∗ Hoogsteen bonding (oligopyrimidine (Y), oligopurine (R) and thrid strand (Z)).

*Corresponding author. Tel.: +33-1-40793711; fax: +33-1-40793705; e-mail: sun@mnhn.fr

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formation when covalently linked to the TFO;[7,8] it is also able to induce Topo I-mediated DNA cleavage[18] as do the indolocarbazole derivatives. Because DNA cleavage by topoisomerase I is not sequence-specific, the linkage of these Topo I poisons to a TFO should direct the cleavage by the enzyme specifically at the triplex site, where the poison is anchored by triplex formation. This has been recently confirmed by the findings of Matteucci et al.,[19] which show that a triple helix-forming oligonucleotide conjugated to camptothecin is able to direct topoisomerase I-mediated DNA cleavage to specific sites.

Firstly, we investigated the ability of indolocarbazole and benzopyridoquinoxaline compounds covalently linked to the TFOs to stabilize triple helices in vitro as compared to the unmodified oligonucleotide. The stability of the triplexes was assessed by polyacrylamide gel electrophoresis (PAGE). The results are discussed with respect to the different attachment sites and functional groups of the drugs. Secondly, we studied by DNase I footprinting whether the structural perturbations of the DNA induced by triplex formation inhibit Topo I binding near the triplex/duplex junction. Thirdly, we investigated whether these conjugates are able to direct DNA cleavage by topoisomerase I at the triplex site and whether the presence of the triple helical structure affects the DNA cleavage pattern by topoisomerase I.

Results

The synthesis of the indolocarbazole R-6[20] and of the benzopyridoquinoxaline BPQ(1256)[8] have been described previously. The R-95 derivative was synthesized as described in Experimental.

Oligonucleotides

The targets chosen for this study were a 29- and a 59-bp duplex containing a 16-bp oligopyrimidine•oligopurine sequence (Fig. 1A). The TUC oligonucleotide containing 5-propynyl-2′-deoxyuracil and 5-methyl-2′-deoxycytosine was shown to form a stable triple helix on this target sequence at 37 °C pH = 7.2, 50 mM KCl, 10 mM $MgCl_2$.[19] These base modifications were used because they were previously shown to enhance triplex formation.[21] We covalently attached the indolocarbazole derivatives R-6 and R-95 to the 3′-end of the oligonucleotide via an hexaethylene glycol linker arm as described in Experimental (Fig. 1B). The benzopyridoquinoxaline derivative, BPQ(1256), was directly attached to the phosphorylated 3′-end of TUC through the NH_2 group of the aliphatic linker chain at position 7 (Fig. 1A). The unmodified TUC was used as a control.

Synthesis of oligonucleotide-drug conjugates

The linkage of drugs such as BPQ(1256) or R-95 to the oligonucleotide with a terminal phosphate through a primary amine function has already been described in detail.[7,8]

In the case of the derivative R-6, the linkage to the oligonucleotide was achieved through the attachment of a ribose (uridine) at the end of the TFO to the hydrazide function of the compound (Fig. 2). First the diol function of the ribose was oxidized with sodium periodate to aldehydic functions. Then the two aldehydic groups reacted with the hydrazide function of R-6 and the conjugate was recovered by HPLC purification.[22,23]

The R-6 derivative was covalently linked via the F cycle, and the R95 derivative via the B cycle. Upon triplex formation, the TFO binds in the major groove of DNA and forms hydrogen bonds with the oligopurine strand of the duplex. Therefore the position of the planar intercalated indolocarbazole derivatives at the triplex/duplex junction is different with the two derivatives R-6 and R-95.

Triplex stabilization

The binding of the third strand to the duplex decreases the electrophoretic mobility of the radiolabeled duplex (Fig. 3). The formation of the triple-helical complex can be quantitated from a phosphorimager analysis of the different species in a gel retardation assay. The affinity of the TFOs for the duplex (K_d) was calculated as described in Experimental. Table 1 summarizes the values of K_d for the oligonucleotide-intercalator conjugates (TFO−drug) and for the unmodified oligonucleotide in the presence of free intercalator (TFO + drug).

The most dramatic stabilization was achieved with the oligonucleotide TUC-R-6 carrying the indolocarbazole R-6 at its 3′-end. This conjugate exhibited a K_d value of 0.20 μM, which means a 26-fold stabilization of the triplex as compared to the unmodified TUC. The conjugate carrying the R-95 derivative shows only a 6-fold stabilization. The BPQ-conjugate forms a slightly stronger triplex than the R-95 conjugate, but a weaker one than the R-6 conjugate. The unlinked R-6 derivative slightly stabilizes the triplex, as does the R-95 derivative. The benzopyridoquinoxaline ligand BPQ(1256), when unlinked, shows the best stabilization (about 3-fold at 10 μM).

Rebeccamycin and benzopyridoquinoxaline derivatives have the ability to induce topoisomerase I-mediated DNA cleavage.[17,18,24] Therefore we investigated the influence of the presence of the triple-helical structure and in particular of the triplex formed by the conjugates on the DNA cleavage pattern induced by human topoisomerase I.

DNA cleavage by topoisomerase I in the presence of triple helix

In the first place, we studied whether triplex formation alone, which induces conformational changes of DNA,[25] affects DNA binding of topoisomerase I.[26] This study was carried out by DNase I footprinting on the 59-bp target duplex, radiolabeled at the 3′-end of the oligopyrimidine-containing strand, and using the

Target duplex:

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5'GATA GAGAGAGAGAAAAAAA GAGAAGATC 3'29R
3'CTAT CTCTCTCTCTTTTTTT CTCTTCTAG 5'29Y

5'CACTCCCTATCAGTGATAGAGAGAGAGAAAAAAAGAGAAGATCTGAGCTCGGTACCCT3' 59R
3'GTGAGGGATAGTCACTATCTCTCTCTCTTTTTTTCTCTTCTAGACTCGAGCCATGGGA5' 59Y
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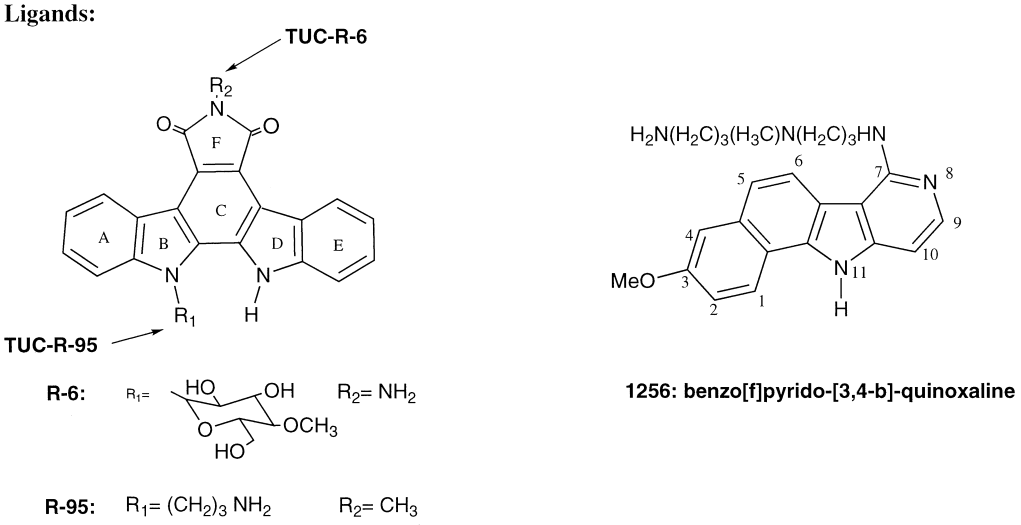
Oligonucleotides:

5' MPMPMPMPMPPPPPPP 3'	TUC
5' MPMPMPMPMPPPPPPP-1256 3'	TUC-BPQ(1256)
5' MPMPMPMPMPPPPPPP-L18-rU-R-6 3'	TUC-R-6
5' MPMPMPMPMPPPPPPP-L18-R-95 3'	TUC-R-95

(a)

Ligands:



(b)

Figure 1. A. Sequence of the TFOs, of the 29-bp target duplex and of the 59-bp target duplex. M = 5-methyl-2′-deoxycytidine, P = 5-propynyl-2′-deoxyuridine. rU = 2′-ribose-uridine, L18 = hexaethylene glycol. B. Structure of the indolocarbazole derivatives and the benzopyridoquinoxaline used in this study. The position of the linkage to the TFO is indicated by an arrow.

topoisomerase I mutant Y723 which contains a phenylalanine in the place of the catalytic tyrosine residue at position 723. This mutant remains capable of forming DNA–topoisomerase I complexes, but it is unable to cleave DNA.[27] Under these conditions, DNA cleavage is only caused by the endonuclease DNase I, used as an accessibility probe. The radiolabeled target duplex was treated in the absence (Fig. 4A, lanes 2 and 3) or in the presence (lanes 4 and 5) of Topo I Y723 and in the absence (lanes 2 and 4) or in the presence (lanes 3 and 5) of the triplex formed with the unmodified TFO(TUC).

A clear footprint reveals triplex formation as can be seen in lane 3. The footprints of the Y723 mutant were observed at the 5′-end of the triplex (lane 4, brackets b), at the 3′-end (brackets c1 and c2) and in the triplex region (brackets a). Upon triplex formation DNase I protection pattern was slightly changed on the 3′-end of the triplex (lane 5). The intensity of the bands changes and a new DNase I cleavage site appears suggesting that the Y723 mutant was shifted further down.

These results correlate well with the DNA cleavage pattern by topoisomerase I on the 59-bp target duplex observed in Figure 4B. The 59-bp target duplex, radiolabeled at the 3′-end of the oligopyrimidine-containing strand, was incubated with topoisomerase I in the absence (lane 1) or in the presence of the poison BPQ(1256) (lanes 2) or of the triplexes (lanes 3 and 4).

Topoisomerase I cleaves in the triple helix region (lanes 1 and 2, brackets a), which is consistent with the protection of this region against DNase I cleavage observed in the presence of the mutant Y723 (Fig. 4A, lane 4). When 5 μM of BPQ(1256) are added, the Topo I cleavage pattern is not changed. Triplex formation protects

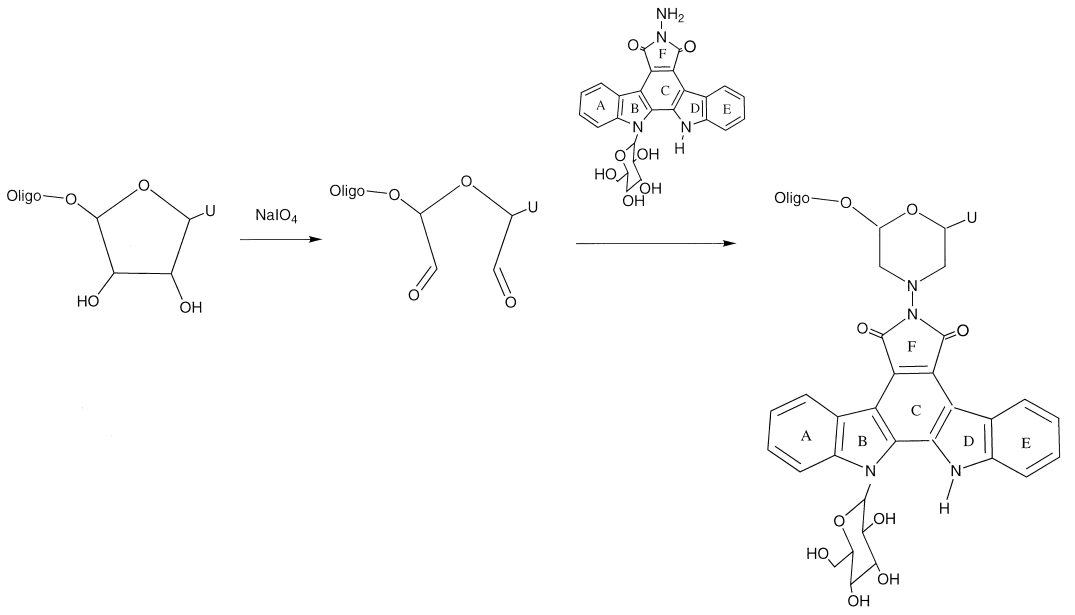


Figure 2. Coupling of the TFO to the R-6 derivative.

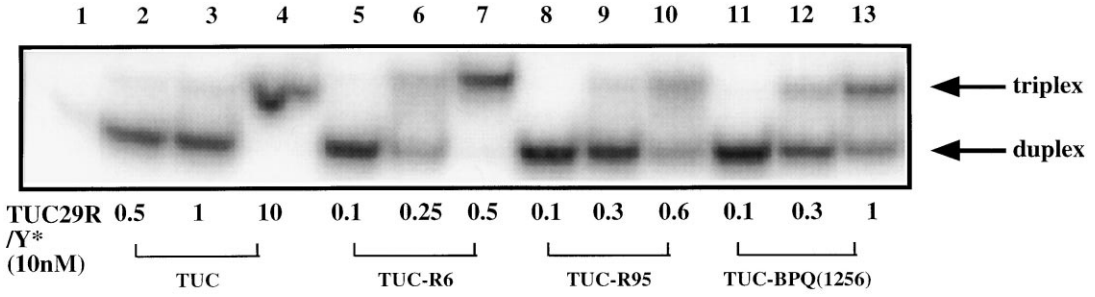


Figure 3. The oligopyrimidine radiolabeled duplex 29R/29Y* (10 nM. was incubated with: increasing concentrations of TFOs in 50 mM HEPES pH = 7.2, 10 mM $MgCl_2$ and 100 mM KCl, at 37 °C. Lane 1, no TFO; lanes 2–4, 0.5, 1 μM and 10 μM, respectively, of TUC were added to 10 nM of duplex; lanes 5–7; 0.1, 0.25 μM and 0.5 μM, respectively, of TUC-R-6 were added; lanes 8-10, 0.1 μM; 0.3 μM and 0.6 μM of TUC-R-95 were added; lanes 11–13, 0.1, 0.3 and 1 μM, respectively, of TUC-BPQ(1256) were added.

Table 1. Dissociation constants (K_d) for the TUC29R/29Y system[a]

TFO	K_d (μM^{-1})	
TUC	5.3 ± 0.5	
	TFO-drug	TFO + drug
TUC/BPQ(1256)	0.73 ± 0.09	1.9 ± 0.1
TUC/R-6	0.20 ± 0.06	3.6 ± 0.9
TUC/R-95	0.85 ± 0.07	4.8 ± 0.3

[a]The dissociation constant (K_d) was calculated as previously described[33] and an average value corresponding to five to eight different experiments is reported. The concentration of the free drug (right column) was 10 μM.

the DNA from topoisomerase I cleavage (lanes 3 and 4) and the strong sites a disappear. An enhanced cleavage is observed on the 3′-end of the triplex (brackets b), where, according to DNase I footprinting experiments, the Topo I is positioned in the presence of the triplex.

BPQ and indolocarbazole derivatives have the ability to induce Topo I-mediated cleavage but in a non sequence-specific manner. Therefore we investigated whether the coupling of these derivatives to a TFO could direct the action of these topoisomerase I poisons specifically at the triplex site. The target duplex was first inserted in plasmid pBSK(±) (Promega). Then a 72-bp fragment containing the oligopyrimidine•oligopurine target sequence was excised from this plasmid and radiolabeled at the 3′ end of the oligopyrimidine strand. Evidence for triplex formation on this fragment was provided by DNase I footprinting experiments (as described in Figure 4 for a shorter fragment). After triplex formation, the samples were incubated 1 h at 37 °C with topoisomerase I, digested by proteinase K/SDS treatment and the cleavage products were analyzed on a 15% denaturing gel. Figure 5 shows that the TUC-R-6 conjugate (lane 7) induced specific cleavage at site b1 at the 3′-end of the triple helix, i.e. at the site where the Topo I poison is attached to the TFO, whereas the free derivative produced cleavage at other sites (R-6, lane 4). Formation of the triple helix inhibited Topo I-induced cleavage on the 5′-side of the triplex site (site c on Fig. 5) and at the b2 site, further away from the 3′-end of the triplex. Furthermore, the yield of cleavage at the b1 site was significantly enhanced when the topoisomerase

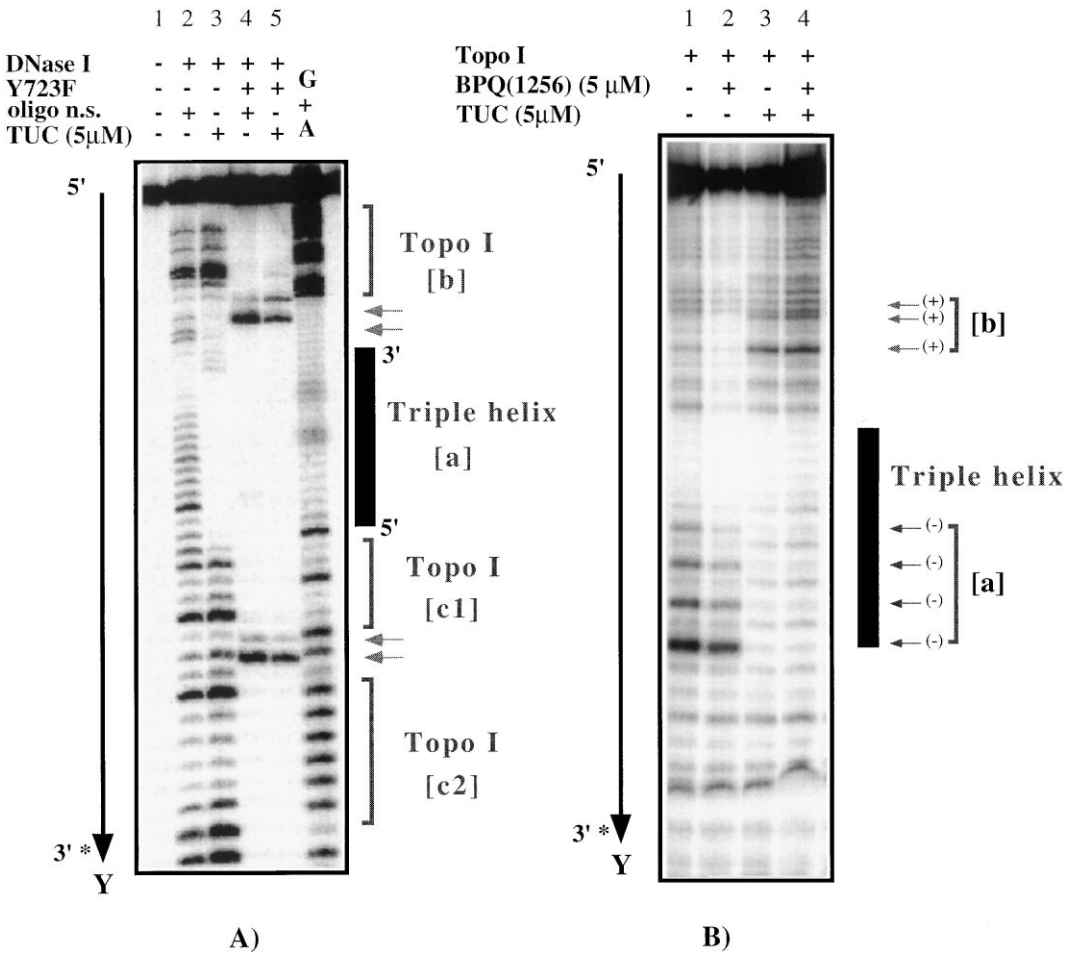


Figure 4. A. DNase I footprinting experiments on the 59-bp target duplex (50 nM), 3′-end radiolabeled on the oligopyrimidine-containing strand. Lane 1, duplex; lane 2, duplex treated with DNase I; lane 3, duplex treated with DNase I in the presence of TUC (5 μM); lane 4, duplex incubated with Topo I mutant Y723F (5 min at 37 °C) and then treated with DNase I; lane 5, duplex incubated with Y723F in the presence of TUC (5 μM) and then treated with DNase I. Adenine/guanine-specific Maxam–Gilbert chemical cleavage reactions were used as markers. The brackets indicate the triplex site and the Y723F binding sites. B. Sequence analysis of the Topo I-mediated cleavage products on the 59-bp target duplex of Figure 1A 3′-end radiolabeled on the oligopyrimidine-containing strand. The brackets mark different cleavage regions and the triplex site. Lane 1, target duplex; duplex incubated with topoisomerase I in the absence (lane 2 or in the presence of 5 μM BPQ(1256) (lane 3, 5 μM TUC (lane 4, 5 μM TUC and 5 μM BPQ(1256) (lane 5).

poison (R-6 or R-95) was attached to the 3′-end of the TFO TUC (Fig. 5, lanes 6 and 7). The efficacy of cleavage was different for the two rebeccamycin conjugates (TUC-R-6, lane 7 and TUC-R-95, lane 6); the TUC-BPQ(1256) led to a very weak cleavage (data not shown). The TUC-R-6 conjugate gave the strongest cleavage, as observed for the free derivative.

Discussion and Conclusion

Two topoisomerase I poisons containing an indolocarbazole moiety were chosen for their different functional groups and attachment sites to the triplex-forming oligonucleotide: the R-6 derivative and the R-95 derivative (Fig. 1). An hexaethylene glycol linker was used between the drug and the oligonucleotide in order to ensure the necessary length and flexibility for the drug to interact with topoisomerase I without interference with triplex formation. A benzopyridoquinoxaline (BPQ) derivative, which has been reported to be both a specific triple helix ligand[7,8] and a topoisomerase I poison,[18] was also attached to the TFO, and compared to the indolocarbazole–TFO conjugates.

Gel shift assays showed that covalent attachment to the 3′-end of a TFO provides significant triplex stabilization (up to 26-fold, Figure 3 and Table 1). It is likely that the observed stabilization originates from the intercalation of the indolocarbazole derivatives at the duplex/triplex junction since it has been reported that indolocarbazole compounds interact with double-stranded DNA by intercalation.[11] The difference in binding affinity between the two indolocarbazole–TFO conjugates could be rationalized, at least in part, by the difference in linkers and attachment sites on the drug, and thus a different location of the drug in the complex with respect to DNA. As a matter of fact, upon triplex formation, the B-cycle and the sugar moiety of the drug in the case of the TUC-R-6 conjugate is located in the minor groove of the target duplex, whereas the B-cycle in the case of the TUC-R-95 conjugate is in the major groove.

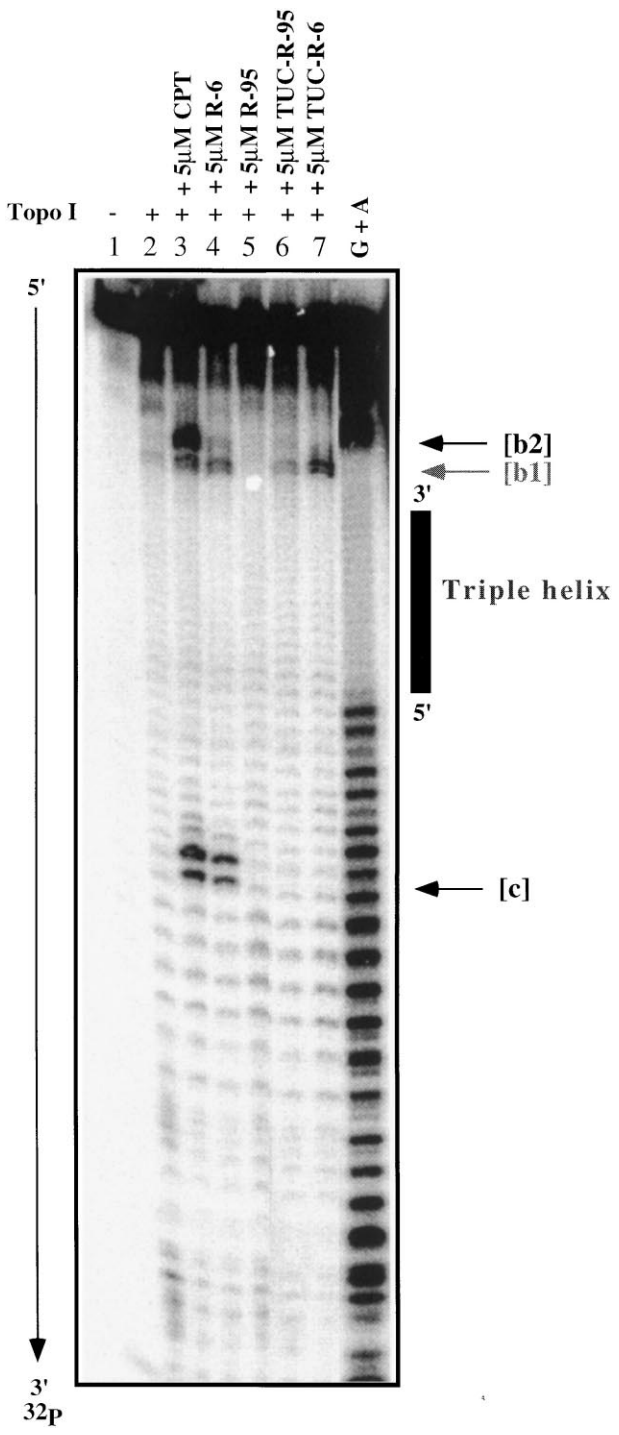


Figure 5. Sequence analysis of the Topo I-mediated cleavage products on the 72-bp target duplex 3′-end radiolabeled on the oligopyrimidine-containing strand. The alphabet letters mark different cleavage sites and a black bar the triplex site. Guanine specific Maxam–Gilbert chemical cleavage reactions were used as markers. Lane 1, target duplex; duplex incubated with topoisomerase I in the absence (lane 2) or in the presence of 5 μM camptothecin (lane 3), 5 μM R-6 (lane 4), 5 μM R-95 (lane 5), 5 μM TUC-R-95 (lane 6), 5 μM TUC-R-6 (lane 7).

A DNase I protection assay using both native Topo I and Topo I mutant (Y723) that still binds DNA but is no more able to cleave within the triplex region was carried out. These observations showed that interference of triplex formation with Topo I binding and DNA cleavage was mainly restricted to the TFO binding site (Fig. 4). The formation of the triple-helical structure alters the accessibility of this region and thus changes the positioning of the topoisomerase I along the DNA fragment.

The indolocarbazole and BPQ derivatives that we have investigated are known to induce topoisomerase I-mediated DNA cleavage in a non sequence-specific manner. It was, therefore, of interest to show that DNA sequence recognition by intermolecular triple helix formation could be exploited to direct the action of these Topo I poisons to specific sites. It was found that the TFO-3′-drug conjugates were able to induce Topo-I mediated DNA cleavage specifically at the 3′-end of the triplex site, in agreement with the recent finding of Matteucci et al. using a camptothecin–TFO conjugate.[19] The yield of cleavage depended on the chemical nature and on the attachment site of Topo I poisons (Fig. 5). The TUC-R-6 conjugate, in which the B-cycle and the sugar moiety of rebeccamycin were expected to be located in the minor groove upon triplex formation and intercalation of R-6, gave the highest cleavage efficacy. This result, in agreement with previous data showing that the location of the sugar moiety in the minor groove, was a determinant factor for Topo I-mediated DNA cleavage.[11,24]

The present proof-of-principle work shows that triplex-directed topoisomerase I-mediated DNA cleavage by using Topo poison conjugated to TFOs offers new perspectives in several fields: (i) these conjugates provide a new approach to understand the interactions involved in the Topo I/drug/DNA ternary complex, by using the advantage that the drug can be located in different and known orientations with respect to topoisomerase and DNA, just by varying the attachment sites and the functional groups as well as the nature and length of the linker; (ii) by linking an hydrophilic oligonucleotide to a rather hydrophobic Topo inhibitor, it is expected that the poor solubility of the drug should be improved and therefore the reflux by MDR-coded membrane pumps could be reduced; (iii) the site-directed DNA cleavage could be used to probe DNA repair and chromatin structures in situ; and (iv) the ability of TFO-conjugates to target Topo I cleavage to specific genes could provide a new paradigm for the development of more specific and less toxic chemotherapeutic agents. Further investigations are currently underway to address these questions.

Experimental

Oligonucleotides

Oligonucleotides were purchased from Eurogentec and purified using quick spin columns with Sephadex G-25 fine (Boehringer, Mannheim). The drug-tethered oligonucleotides were synthesised as described below. Concentrations of oligonucleotides were determined spectrophotometrically at 25 °C using molar extinction coefficients at 260 nm calculated from a nearest-neighbor model.[28]

Drugs

The synthesis of the indolocarbazoles R-6[20,29] and of the benzopyridoquinoxaline BPQ(1256)[8] have been described previously. Ligand concentrations were measured by weighing. All the drugs were dissolved in dimethylsulfoxide at 3 mg/mL and then diluted further with water. Fresh dilutions were prepared immediately before use. The final dimethylsulfoxide concentration in the studies of triplex formation or cleavage experiments never exceeded 0.3% (v/v).

The 6-methyl-13-*N*-(1,3-diamminopropyl)-5,7-dioxo-(5H)-6,7,12,13-tetrahydro-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole (R-95) derivative. To a solution of *N*-methylated aglycone 6-methyl-5,7-dioxo-(5H)-tetrahydro-indolo[2,3-*a*]-pyrrolo[3,4-*c*]-carbazole[30] (400 mg, 1.16 mmol) in DMF (50 mL) was added a suspension of NaH (60% dispersion in mineral oil, 140 mg) in DMF (15 mL). The mixture was stirred at room temperature for 30 min before the addition of a solution of 3-bromopropylamine hydrobromide (260 mg, 1.18 mmol) in DMF (15 mL). The mixture was stirred at room temperature for 24 h. After addition of saturated aqueous $NaHCO_3$ and extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue purified by flash chromatography (silicagel, eluent CH_2Cl_2:MeOH, 95:5) to give the amine R-95 (217 mg, 0.55 mmol, 47% yield) as a yellow solid. Mp > 300 °C. IR (KBr) ν_{CO} 1695, 1745 cm^{-1}, ν_{NH} 3100–3600 cm^{-1}. HRMS (FAB+) $(M+H)^+$ calculated for $C_{24}H_{21}N_4O_2$ 397.1664, found 397.1647.

^1H NMR (400 MHz, DMSO-d_6): 2.15 (2H, m), 2.75 (2H, t, J = 5.4 Hz), 3.16 (3H,s, NCH_3), 3.39–4.01 (3H, br, s), 4.87 (2H, t, J = 6.4 Hz), 7.37 (1H, dt, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 7.41 (1H, t, J = 7.9 Hz), 7.58 (1H, dt, J_1 = 7.9 Hz, J_2 = 1.0 Hz), 7.63 (1H, t, J_1 = 7.4 Hz, J_2 = 0.9 Hz), 7.75 (1H, d, J = 7.9 Hz), 7.83 (1H, d, J = 8.3 Hz), 9.08 (1H, d, J = 7.9 Hz), 9;11 (1H, d, J = 7.9 Hz).

^{13}C NMR (100 MHz, DMSO-d_6): 23.4 (NCH_3), 31.4, 36.7, 41.3 (CH_2), 109.7, 112.2, 119.7, 120.1, 124.1, 124.3, 126.5, 126.6 (C tert arom), 115.2, 116.8, 118.2, 118.6, 121.0, 121.1, 129.3, 130.1, 141.0, 141.9 (C quat arom), 169.6 (2 C=O).

Synthesis of the TFO-conjugates

The TUC-R-95 and TUC-BPQ(1256) conjugates. We covalently linked the NH_2 group of the aliphatic chain of R-95 to the phosphorylated hexaethylene glycol linker arm at the 3′ end of TUC or the NH_2 group of the aliphatic chain of BPQ(1256) derivative (Fig. 1B) to the 3′-end of the phosphorylated TUC.[31,32] 150 μg of 3′ phosphorylated oligonucleotide (Fig. 1) was first precipitated as hexadecyltrimethylammonium salt and the oligonucleotide salt was then dissolved in 50 μL of dry DMSO. A 5 μL sample of *N*-methylimidazole and 25 μL each of dipyridyl disulfide and triphenylphosphine solutions (1.2 M DMSO) were added. After 15 min incubation at room temperature, 5 μL of triethylamine was added followed by the drug solution (20 μL, 30 mM in DMSO). After 20 min the oligonucleotide was precipitated with $LiClO_4$ and purified by reverse phase HPLC using a linear acetonitrile gradient (0–80% CH_3CN in 0.2M $(NH_4)OAc$). Average yield 60%. The oligonucleotide–ligand conjugates were characterized by UV spectroscopy.

The TUC-R-6 conjugate. To covalently link R-6 to the 3′-end of the oligonucleotide we used the 16-mer TUC bearing at the 3′-end an uridine linked through an hexaethylene glycol linker arm. 300 nmols of oligonucleotide, previously precipitated with ethanol, and 6.5 mg of sodium periodate ($NaIO_4$) were dissolved in 100 μL of 200 mM sodium acetate, pH = 5.2. After 20 min incubation at room temperature and in the dark, 100 μL of potassium chloride 2 M were added in order to precipitate the excess of periodate. The pellet was washed several times with 50 μL of H_2O. All the water phases were collected and precipitated with ethanol in order to collect the oligonucleotide and to eliminate KCl. The oligonucleotide was dissolved in 50 μL of 200 mM NaOAc, pH = 5.2 and was added to 1 mg of R-6 or R-0 dissolved in 50 μL DMF for 12 h at 4 °C in the dark. After ethanol precipitation, the oligonucleotide was recovered by reverse phase HPLC using a linear acetonitrile gradient (0–80% CH_3CN in 0.2 M $(NH_4)OAc$). Average yield 40%. The oligonucleotide–ligand conjugates were characterized by UV spectroscopy.

Gel retardation assay

The oligopyrimidine strand of the duplex (Fig. 1A) was 5′ end-labeled with [γ-^{32}P] ATP (Amersham Arlington Heights, IL) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

Increasing concentrations (100 nM–10 μM) of the triplex-forming oligonucleotides were added to 10 nM of the radiolabeled duplex in the absence or in the presence of 10 μM of drugs, in 10 mM $MgCl_2$, 100 mM KCl, 50 mM HEPES, pH 7.2, 10% sucrose and 0.5 mg/mL tRNA with sample incubation at 37 °C overnight. Electrophoresis was performed on a non-denaturing 12% polyacrylamide gel containing 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.2, at 37 °C. The dissociation constant (K_d) was calculated as previously described [33] and a mean value corresponding to five to eight different experiments is reported.

DNase I protection assays

The 59-mer oligopyrimidine strand was 3′ end-labeled with [α-^{32}P] ddATP (Amersham Arlington Heights, IL) by terminal transferase (Amersham Arlington Heights, IL) and was incubated 1 h at 37 °C, in 50 mM Tris–HCl, pH = 7.5, 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, in the presence of the TFO (at the indicated concentration) in order to form the triplex (total reaction volume 20 μL). 1 μL of DNase I (0.2 mg/mL final, Sigma, Germany) diluted in 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl, was added to the radiolabeled duplex, preincubated 1 h at 37 °C in the buffer described above in the absence or in the presence of TFO. The reaction was

performed for 4 min at 20 °C. The reaction was stopped by the addition of 1.5 μL solution containing 1 mM EDTA and 1.25% SDS and vigorous stirring. After double precipitation in ethanol the samples were resuspended in 95% formamide and heated at 95 °C for 4 min before being loaded onto a denaturing 15% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in TBE buffer (50 mM Tris base, 55 mM boric acid, 1 mM EDTA).

Topoisomerase I cleavage assays

The DNA template radiolabeled on the oligopyrimidine-containing strand of the target was obtained as described above. To analyze the Topo I DNA cleavage products, 10 units of enzyme were added to the duplex, preincubated in 50 mM Tris–HCl, pH = 7.5, 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 μg/μl BSA with either the TFO or/and the drugs, followed by 1 h incubation at 37 °C. The DNA–topoisomerase I cleavage complexes were dissociated by addition of SDS to a final concentration of 0.25% and of proteinase K (Sigma) to 250 μg/mL, followed by incubation for 35 min at 55 °C. After ethanol precipitation, all samples were resuspended in 6 μL of formamide, heated at 90 °C for 4 min and then chilled on ice for 4 min, before being loaded onto a denaturing 15% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in 1×TBE buffer. To quantitate the extent of cleavage, the gels were scanned with a Molecular Dynamics 445SI Phosphorimager. For the experiment described in Figure 5, the 59-bp target duplex was inserted between the BamH I and EcoR I sites of plasmid pBSK(±) (Promega). The digestion of the plasmid by BamH I and Eco RI yielded a 72-mer fragment suitable for 3′-end labeling on the oligopyrimdine-containing strand by the Klenow polymerase. The detailed procedures for isolation, purification and labeling of this duplex DNA fragment have been described previously.[34]

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