Design and Optimization of Camptothecin Conjugates of Triple Helix-forming Oligonucleotides for Sequence-specific DNA Cleavage by Topoisomerase I*

Paola B. Arimondo‡‡, Alexandre Boutorine§, Brigitte Baldeyrou®, Christian Bailly¶, Masayasuke Kuwahara*, Sidney M. Hecht†, Jian-Sheng Sun‡, Thérèse Garestier‡, and Claude Hélène‡

From the ½Laboratoire de Biophysique, UMR 8646 CNRS, Museum National d’Histoire Naturelle, INSERM U201, 43 rue Cuvier, 75231 Paris cedex 05, France, ½INSERM U824 and Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, Institut de Recherches sur le Cancer de Lille, Place Verdun, 59045 Lille, France, and the ½Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901

To achieve a sequence-specific DNA cleavage by topoisomerase I, derivatives of the antitumor drug camptothecin have been covalently linked to triple helix-forming oligonucleotides that bind in a sequence-specific manner to the major groove of double-helical DNA. Triplex formation at the target sequence positions the drug selectively at the triplex site, thereby stimulating topoisomerase I-mediated DNA cleavage at this site. In a continuous effort to optimize this strategy, a broad set of conjugates consisting of (i) 16–20-base-long oligonucleotides, (ii) alkyl linkers of variable length, and (iii) camptothecin derivatives substituted on the A or B quinoline ring were designed and synthesized. Analysis of the cleavage sites at nucleotide resolution reveals that the specificity and efficacy of cleavage depends markedly on the length of both the triple-helical structure and the linker between the oligonucleotide and the poison. The optimized hybrid molecules induced strong and highly specific cleavage at a site adjacent to the triplex. Furthermore, the drug-stabilized DNA-topoisomerase I cleavage complexes were shown to be more resistant to salt-induced reversal than the complexes induced by camptothecin alone. Such rationally designed camptothecin conjugates could provide useful antitumor drugs directed selectively against genes bearing the targeted triplex binding site. In addition, they represent a powerful tool to probe the molecular interactions in the DNA-topoisomerase I complex.

The reaction between double-stranded DNA and topoisomerase I produces a covalent 3’-phosphorytyrosyl adduct, usually referred to as the cleavage complex (1, 2). Under physiological conditions, the covalent intermediate is barely detectable, because a fast religation step occurs after relaxation of the DNA constraints. A number of drugs, such as the antitumor alkaloid camptothecin (CPT),1 can convert topoisomerase I into a cell poison by blocking the religation step, thereby enhancing the formation of persistent DNA breaks responsible for cell death (1, 3, 4). However, topoisomerase I poisons display a weak sequence specificity. Mainly one or two nucleotides on the 3’ and 5’-side of the cleavage site (essentially thymine-guanine steps in the case of CPT) represent the only recognition elements. As a result, drugs like CPT induce massive nonspecific DNA damage in cells and can affect any gene within the genome. The identification, over the last decade, of genes that play a key role in the progression and maintenance of a specific disease, such as oncogenes and tumor suppressor, calls for the development of drugs able to regulate the expression and functions of these genes. For this reason, the design of molecules that bind to specific sequences in DNA is urgently needed. To direct the topoisomerase I enzymatic reaction to particular sites, topoisomerase I poisons, including camptothecin and rebeccamycin derivatives, have been covalently attached to sequence-specific DNA ligands, such as triplex-forming oligonucleotides (TFO) (5–7) and hairpin polyamides (8, 9) that bind in a sequence-specific manner to the major and minor groove of double-helical DNA, respectively.

Interesting results have been obtained previously with triplex-forming oligonucleotide-camptothecin conjugates (TFO-CPT) (5, 6), but from initial attempts to direct topoisomerase I-mediated cleavage to a specific site, it was clear that the system had to be carefully optimized to enhance the specificity and efficacy of cleavage (7). We report here on the effect of the length of the triple-helical structure and the linker arm between the TFO and the poison, as well as the influence of the drug orientation with respect to the topoisomerase I cleavage site. The DNA binding and topoisomerase I-mediated cleaving properties of the conjugates were investigated by PAGE at nucleotide resolution, and their effects on the stability of the topoisomerase I-DNA complexes were also investigated. Altogether the results attest unequivocally that the recognition and cleavage of DNA by topoisomerase I can be fully controlled by the attachment of a CPT-type poison to a TFO, but the structure of the hybrid molecule must be precisely adapted to direct the cleavage reaction to the target site.

EXPERIMENTAL PROCEDURES

Oligonucleotides and DNA Fragment

Oligonucleotides were purchased from Eurogentec and purified using quick spin columns and Sephadex G-25 fine (Roche Molecular Biochemicals). Camptothecin, camptothecin-DNA, and deoxyribonuclease I.
The nomenclature of the oligonucleotides and conjugates is as follows. The abbreviation TFO is followed by a number referring to the length of the oligonucleotide, followed by the letter L (for linker) and the number of carbon atoms in the linker, and finally, by the denomination of the camptothecin derivative (10CPT for 10-camcamptothecin or 7CPT for 7-(2-aminoethyl)camptothecin). For example, TFO20-L4-10CPT stands for the 20-mer TFO linked through the diamino butyl spacer suitable for 3'-end labeling by the Klenow polymerase and 10CPT stands for the 20-mer TFO linked through the diaminobutyl spacer to 10-camcamptothecin.

The plasmid pBSK (+/-) was bought from Promega, and the 77-bp target duplex was inserted between the BamHI and EcoRI sites. The digestion of the plasmid by PstII and EcoRI yielded a 324-mer fragment suitable for 3'-end labeling by the Klenow polymerase and [γ-32P]ATP (Amersham Biosciences, Inc.). The detailed procedures for isolation, purification, and labeling of this duplex DNA fragment have been previously described (11).

**Topoisomerase Poisons**

All the drugs were dissolved in dimethyl sulfoxide at 3 mg/ml and then added further with water. The final dimethyl sulfoxide concentration never exceeded 0.3% (v/v) in all assays. 10-Carboxycamptothecin (kindly provided by Dr. Mark Matteucci, Gilead Sciences; structure in Fig. 2) was conjugated to the terminal amino group of a diaminoalkyl linker arm at the 3'-end of the oligonucleotide as described in Ref. 6. The linker arm was attached by reaction of the corresponding alkylamine to the 3'-phosphorylated oligonucleotide activated by N,N'-dipropyldisulfide and triphenylphosphine as described in Ref. 12.

**7-(2-Aminoethyl)camptothecin**

Synthesis—7-(2-Aminoethyl)camptothecin 7CPT (5) was obtained by synthetic transformation of the natural product camptothecin (1) as outlined in Fig. 1.

**Fig. 1. Synthetic route used for the preparation of 7-(2-aminoethyl)camptothecin 7CPT.**

7-(2-Hydroxyethyl)camptothecin (2)—To a suspension of 100 mg (0.29 mmol) of camptothecin and 160 mg (0.58 mmol) of P2O5, 7.5 ml of EtOH, 2 ml of CHCl3, and 4 ml of H2O was added dropwise 2 ml of H2SO4. This suspension was cooled to 0°C and then stirred and treated dropwise with 1 ml of 30% H2O2. The reaction mixture was stirred at room temperature for 3 h and then diluted with water, which afforded a precipitate. The precipitate, which consisted mostly of CPT and 7-methyl CPT in addition to the desired product, was triturated with 10% EtOH in CHCl3 and the combined extract was concentrated to afford the crude product. This material was purified by chromatography on silica gel; elution was effected with MeOH-CHCl3 mixtures (1:99 MeOH-CHCl3 to 3:97 MeOH-CHCl3). The desired 7-(2-hydroxyethyl)CPT (2) crystallized from ethanol as a colorless microcrystalline solid; yield, 19 mg (17%); silica gel Tlc TLC Rf = 0.09 (7.5 CHCl3-acetone); mass spectrum (chemical ionization), m/z 392 (M+H)+.

7-(2-P-toluenesulfonyl)camptothecin (2B)—A solution containing 164 mg (0.42 mmol) of 7-(2-hydroxyethyl)camptothecin (2) in 2 ml of pyridine at 4°C was treated with 120 mg (0.63 mmol) of recrystallized p-toluenesulfonyl chloride. The reaction mixture was stirred under argon at 4°C for 20 h and then concentrated under diminished pressure. The residue was dissolved in CHCl3, and the organic phase was washed successively with 0.8 M aqueous citric acid and water and then dried (Na2SO4) and concentrated under diminished pressure. The crude product was purified on a silica gel column (8×1.3 cm); elution was carried out with 1% MeOH in CHCl3. This afforded 7-(2-p-toluenesulfonyloxyethyl)CPT (3) as a pale yellow powder, contaminated with a small amount of 7-(2-chloroethyl)CPT; yield, 214 mg (94%); silica gel Tlc TLC Rf = 0.29 (7.5 CHCl3-acetone); 1H NMR (CDCl3) δ 1.04 (3H, t, 3H), 1.90 (m, 2H), 2.28 (s, 3H), 3.45 (t, 2H), 3.57 (s, 1H), 3.76 (t, 2H), 7.62 (t, 1H), 7.85 (m, 2H), 7.91 (1H, 7.89 (d, 1H), and 8.20 (d, 1H); 13C NMR (CDCl3) δ 7.8, 21.6, 29.4, 31.6, 49.7, 66.3, 68.2, 72.7, 79.1, 118.9, 122.8, 126.7, 127.2, 128.2, 128.9, 129.5, 130.3, 130.7, 131.9, 138.0, 145.0, 146.4, 149.1, 150.1, 151.6, 157.4, and 173.8; mass spectrum (chemical ionization), m/z 347.2 (M+H)+.

7-(2-Azidoethyl)camptothecin (4)—A solution containing 214 mg (0.39 mmol) of 7-(2-p-toluenesulfonyloxyethyl)CPT (3) in 2 ml of N,N-dimethylformamide was treated with 254 mg (3.91 mmol) of sodium azide. The reaction mixture was stirred under argon at room temperature for 24 h and then concentrated under diminished pressure. The residue was suspended in CHCl3, filtered to remove unreacted sodium azide, and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (8×1.3 cm); elution was carried out with CHCl3-acetone mixtures (1% EtOH in CHCl3) to afford 7-(2-azidoethyl)CPT (4) contaminated with a small amount of 7-vinylcamptothecin: yield 141 mg (86%); silica gel Tlc TLC Rf = 0.15 (7.5 CHCl3-acetone); 1H NMR (CDCl3) δ 1.02 (3H, t, 3H), 1.88 (m, 2H), 3.41 (m, 2H), 3.77 (br, 1H), 3.82 (t, 2H), 3.54 (s, 2H), 5.25 (AB quartet, 2H), 6.85 (d, 2H), 7.32 (d, 2H), 7.62 (t, 1H), 7.85 (m, 2H), 7.91 (1H, 7.79 (1H, 7.89 (d, 1H), and 8.20 (d, 1H); 13C NMR (CDCl3) δ 7.8, 21.6, 29.4, 31.6, 49.7, 66.3, 68.2, 72.7, 79.1, 118.9, 122.8, 126.7, 127.2, 128.2, 128.9, 129.5, 130.3, 130.7, 131.9, 138.0, 145.0, 146.4, 149.1, 150.1, 151.6, 157.4, and 173.8; mass spectrum (chemical ionization), m/z 369.3 (M+H)+.

7-(2-Aminoethyl)camptothecin 7CPT (5)—A solution containing 141 mg (0.34 mmol) of 7-(2-azidoethyl)CPT (4) and 94 mg of 10% palladium-on-carbon in 28 ml of EtOH and 1.1 ml of concentrated HCl was stirred under a H2 atmosphere at room temperature for 20 h. The reaction mixture was filtered through Celite to remove the catalyst, and then the filtered was washed with CHCl3 to remove by-product 7-ethylCPT, and then the aqueous solution was concentrated to dryness under diminished pressure. The residue was purified by chromatography on a silica gel column (8×1.3 cm); elution was carried out with MeOH-CHCl3 mixtures (1% to 40% MeOH in CHCl3) to afford 7-(2-aminoethyl)CPT (5) as a pale yellow powder; yield 50 mg (38%); silica gel Tlc TLC Rf = 0.11 (7.5 CHCl3-MeOH); 1H NMR (MeSO-d6) δ 0.86 (t, 3H), 1.86 (m, 2H), 3.15 (m, 2H), 3.60 (m, 2H), 5.39 (s, 1H), 5.44 (s, 2H), 6.55 (br, 1H), 7.34 (s, 1H), 7.76 (t, 1H), 7.88 (t, 1H), 8.19 (d, 1H), 8.43 (d, 1H), and 8.49 (br, 2H); 13C NMR (MeSO-d6) δ 7.8, 27.1, 30.3, 38.1, 50.0, 65.3, 72.4, 96.8, 118.9, 124.1, 126.9, 128.0, 129.9, 130.0, 130.2, 132.1, 145.9, 148.5, 150.1, 151.6, 157.6, and 173.9; mass spectrum (chemical ionization), m/z 418 (M+H)+.

Conjugation to the TFOs—150 μg of 3'-phosphorylated oligonucleotides, TFO16 and TFO18 (see Fig. 2), were conjugated to the 7CPT via N,N'-dipropyldisulfide and triphenylphosphine solutions (25 μl each 1.2 μM in Me2SO) were added. After 15 min of incubation at room temperature, the activated oligonucleotide was precipitated with...
2% LiClO₄ in acetone, rinsed with acetone, and dissolved in 50 μl of aqueous solution of γ-aminocaproic acid (6 mg) with 5 μl of triethylamine. After 2 h of incubation the oligonucleotide was precipitated with LiClO₄ in acetone, followed by precipitation with ethanol. The terminal carboxyl group of the linker attached to the 3’-end of the oligonucleotides was again activated with dipyridyl disulfide/triphenylphosphine and is underlined for the 16-nucleotide TFO. The 77-bp duplex target sequence was inserted between the BamHI and EcoRI sites of pBSK(+)−). M, 5-methyl-2’-deoxycytidine; P, 5-propynyl-2’-deoxyuridine. The structures of the camptothecin-TFO derivatives used in this study are also shown. The 10-carboxycamptothecin was conjugated to the 3’-end of the TFOs through diamino linkers of different length. The 7-(2-aminoethyl)camptothecin was linked to the 3’-end of TFO16 and TFO18 through γ-aminocaproic acid.

Topoisomerase I Cleavage Assays

The radiolabeled 324-bp target duplex (50 nm) was incubated for 1 h at 30 °C, in 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 30 μg of bovine serum albumin, in the presence of the TFO (at the indicated concentration) to form the triplex (total reaction volume, 20 μl). To analyze the topoisomerase I DNA cleavage products, 10 units of enzyme (Invitrogen) were added to the duplex, preincubated as described above with either the TFO or the drugs, and incubated for 30 min at 30 °C. The DNA-topoisomerase I triplex cleavage complexes were dissociated by the addition of SDS (final concentration, 0.25%) and of proteinase K (Sigma) to 250 μg/ml, followed by incubation for 35 min at 56 °C. After ethanol precipitation, all of the 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 8% polyacrylamide gel (19:1 acrylamide:biacrylamide) containing 7.5 M urea in 1 x TBE buffer (50 mM Tris base, 55 mM boric acid, 1 mM EDTA). To quantitate the extent of cleavage, the gels were scanned with a Molecular Dynamics 445SI PhosphorImager. For the determination of cleavage levels, normalization relative to total loading was performed. To investigate the reversal of the drug-induced cleavage complexes, after incubation with topoisomerase I and prior addition of SDS and proteinase K, increasing concentrations of NaCl were added as indicated for 5 min. The samples were then processed as described above. The bases of the 3’ and 5’ termini of the analyzed cleavage sites were numbered −1 and +1, respectively.

DNase I Protection Assays

The DNA template for the DNase I protection assay on the oligopyrimidine-containing strand of the target was obtained as described above. 1 μl of DNase I (final concentration, 0.2 mg/ml; Sigma) diluted in 1 mM MgCl₂, 1 mM MnCl₂, and 20 mM NaCl was added to the radiolabeled duplex and preincubated for 1 h at 30 °C in the buffer described above in the absence or presence of TFO. The reaction and analysis were performed as described in Ref. 7.

RESULTS

The synthesis of 7-(2-aminomethyl)CPT (7CPT), a new CPT derivative required for the preparation of TFO-CPT conjugates attached to the alkalioid via the 7-position, was carried out as outlined in Fig. 1 starting from camptothecin itself. Thus treatment of CPT (1) with Fe₂O₄, H₂O₂, and H₂SO₄ in aqueous EtOH afforded 7-(2-hydroxyethyl)CPT (2), as described previously (13). Following activation of the primary OH group as a tosylate, treatment with sodium azide in N,N-dimethylformamide afforded 7-(2-azidoethyl)CPT (4) in 81% overall yield from hydroxysylCPT derivative 2. Hydrogenolysis then provided the 7-(2-aminomethyl)camptothecin (7CPT (5)).

The target DNA sequence used in this study and the newly designed TFO-CPT conjugates are shown in Fig. 2. The 77-bp target duplex, containing a central 23-bp oligopyrimidine-oligouridine target sequence for triplex formation, was inserted between the BamHI and EcoRI sites of plasmid pBSK(+)−), and a resulting 324-mer 3’-end 32P-radiolabeled fragment was used for topoisomerase I DNA cleavage and DNA binding assays. We have used this system previously to study triplex formation with 16-nucleotide-long oligomers (underlined and bold in Fig. 2) (6, 7, 14). The TFO moiety of the conjugates consists of 16–20-nucleotide-long oligomers (TFO) containing 5-methyldeoxycytosines and 5-propynyldeoxyuracils to increase triplex stability (15). The spacer corresponds to an alkyl linker containing 4–10 methylene units. The site of attachment of the drug was also varied by the use of camptothecin derivatives substituted on either the A or the B ring of the quinoline moiety, for comparison, 10-carboxycamptothecin and 7-aminomethylcamptothecin derivatives were attached to the TFO via hexyl linkers.

Effect of the Triplex Size and Its Positioning with Respect to Topoisomerase I Cleavage Sites—We investigated how the increase in length of the TFO by two nucleotides on the 3’-side would affect topoisomerase I-mediated DNA cleavage in the proximity of the triplex site by using the previously described 16-mer, TFO16 (5, 6); an 18-mer, TFO18; and a 20-mer, TFO20 (Fig. 2). Even though the potential triplex site contains a 23-bp oligopyrimidine-oligouridine sequence, we limited our study to the 20-mer because a longer oligonucleotide would overlap the strong CPT-induced cleavage site b2 determined previously (6). In a first set of experiments, the 324-bp restriction fragment labeled at the 3’-end of the oligopyrimidine-containing strand was incubated with the TFOs and subjected to limited DNase I cleavage (Fig. 3). As expected, the DNase I cleavage is strongly inhibited at the target oligopyrimidine-oligouridine tract, and the footprint is clearly extended with the 18- and 20-mer TFOs.

Fig. 2. The sequence of the target and of the TFOs used in this study. The TFO binds in the major groove in a parallel orientation to the oligopyrimidine strand of the duplex. The target site is in bold and is underlined for the 16-nucleotide TFO. The 77-bp duplex target sequence was inserted between the BamHI and EcoRI sites of pBSK(+)−). M, 5-methyl-2’-deoxycytidine; P, 5-propynyl-2’-deoxyuridine. The structures of the camptothecin-TFO derivatives used in this study are also shown. The 10-carboxycamptothecin was conjugated to the 3’-end of the TFOs through diamino linkers of different length. The 7-(2-aminoethyl)camptothecin was linked to the 3’-end of TFO16 and TFO18 through γ-aminocaproic acid.
Triplex-directed DNA Cleavage by Topoisomerase I

Fig. 3. Effect of the triplex size with respect to topoisomerase I cleavage. Sequence analysis of the topoisomerase I-mediated cleavage products on the 324-bp target duplex (50 mM 3'-end radiolabeled on the oligopyrimidine-containing strand. Cleavage products were resolved on an 8% polyacrylamide gel containing 7 M urea. Adenine/guanine-specific Maxam-Gilbert chemical cleavage reactions were used as markers (lane G-A). The positions of the cleavage sites are indicated (sites a–f), together with the triple helix region. Note that the 324-bp DNA fragment contained low amounts of shorter fragments before any DNA cleavage reaction (lane DNA). Lane 1, target duplex. Duplex incubated with topoisomerase I (10 units) in the absence (lane 2) or in the presence of 5 μM 10CPT (lane 3), 5 μM TFO16 + 5 μM 10CPT (lane 4), 5 μM TFO18 + 5 μM 10CPT (lane 5), 5 μM TFO20 + 5 μM 10CPT (lane 6), 5 μM TFO16 (lane 7), 5 μM TFO18 (lane 8), or 5 μM TFO20 (lane 9) is shown. The duplex target was digested with DNase I alone (lane 10) or after triplex formation with oligonucleotides TFO16 (lane 11), TFO18 (lane 12), and TFO20 (lane 13) at 5 μM.

(lanes 12 and 13) compared with the 16-mer (lane 11). In parallel experiments, the duplex DNA target was incubated with the 16-, 18-, and 20-mer TFOs prior to cleavage by topoisomerase I (Fig. 3). The reactions were performed in the presence (lanes 4–6) or absence (lanes 7–9) of camptothecin. In the presence of 10-carboxycamptothecin alone (lane 3), strong topoisomerase I-mediated cleavage sites could be detected both on the 3'-side of the target region (at b1 and b2 sites, at 4 and 7 bp from the 16-mer triplex termini, respectively) and on the 5'-side of the target region (site a, at 8-bp from the triple helix end). Additional sites marked c, d, e, and f were also detected. The presence of the 16-mer TFO did not greatly affect the 10CPT-stimulated cleavage (lane 4); only a decrease in cleavage at site a was detected. As the length of the triplex increased on the 3'-ends (lanes 5 and 6), cleavage at sites b1 and b2 gradually decreased, whereas cleavages at sites a and c–f were not significantly altered. A new and weak cleavage site, designated b3, appeared in the presence of the 20-mer TFO (lane 6). A small effect was observed in the presence of the triple helix alone (lanes 7–9), but again, in the presence of the 20-mer TFO, the weak site b3 appeared, and cleavage at site b1 was abolished.

Optimization of the Linker Arm—An important parameter that must be adjusted to optimize topoisomerase I-induced cleavage at the 3'-end of the triple helix is the length of the linker arm between the oligonucleotide and camptothecin. To evaluate this parameter, 10CPT was attached to the TFO16 analog (TFO16-L4–10CPT) (Fig. 2). The spacer was first attached to the 3'-phosphorylated TFO upon activation by dipyridyl disulfide and triphenylphosphine in the presence of N-methylimidazole, and then the other terminal NH2 group of the linker was reacted with the N-hydroxysuccinimide-activated ester of 10-carboxycamptothecin.

Fig. 4 shows the analysis of the topoisomerase I cleavage products on the radiolabeled 324-nucleotide duplex target. All of the conjugates strictly restricted DNA cleavage by topoisomerase I to the 3'-side of the duplex/triplex junction (sites b1 and b2). Cleavage at sites a and c–f completely disappeared. This is a strong indication that the enzyme has been targeted to a specific region, as expected. As the length of the spacer increases, the efficacy of cleavage at site b1 by the TFO16–10CPT conjugate decreases; cleavage is still pronounced with the L6 conjugate (lane 5) but is much poorer with the L10 analog (lane 6).

To delineate further how the presence of the triple helix
influences triplex-directed topoisomerase I-mediated DNA cleavage, we then used triplexes of different lengths in combination with different spacers. Because the conjugate containing the longest linker L10 (TFO16-L10–10CPT) is the least potent at inducing cleavage at site b1 and, furthermore, induces some cleavage at site b2 located at 7 bp from the 3′ triplex end, we restricted the study to the diaminobutane (L4) and diaminohexane (L6) spacers.

**Effect of the Triplex Size and Length of the Linker Arm**—The capacity of the 16-, 18-, and 20-mer TFO-L4/6–10CPT conjugates to induce sequence-specific topoisomerase I-mediated DNA cleavage was compared in Fig. 5. Again DNA cleavage by topoisomerase I was restricted to the 3′-side of the duplex/triplex junction (sites b1 and b2). In the 16-mer conjugates series (lanes 4 and 5), the cleavage efficiency of topoisomerase I at site b1 decreased as the length of the linker increased (as discussed above). The reverse situation was observed in the 18-mer series (lanes 6 and 7). In this case, we detected a much stronger cleavage at site b1 in the presence of the conjugate containing the L6 linker than the analog containing the L4 linker. However, the 18-mer TFO18-L6–10CPT conjugate not only recruited topoisomerase I at site b1 but also promoted cleavage at the adjacent site b2, whereas the 16-mer conjugates stimulated DNA cleavage only at site b1. As in the presence of the triple helix alone (Fig. 3), the 20-mer conjugates strongly reduced the access of topoisomerase I to site b1, and they stimulated cleavage only at the adjacent site b2. Of the 20-mer conjugates, the one containing the hexamethylene linker was the most efficient.

The recruitment of topoisomerase I to sites b1 or b2 is markedly dependent on the length of the TFO and/or linker. In all cases, cleavage remained specific to the proximity of the 3′ triplex end; the figure clearly shows the loss of cleavage at site a and at sites c–f. All conjugates form triple helices, as revealed by footprinting experiments (data not shown).

**Influence of the Camptothecin Moiety**—The experiments described above as well as the studies previously reported by us (6) and others (5) were all performed with a CPT derivative substituted on the A ring. Here we extended the study to a B ring-substituted analog. According to the topoisomerase I-DNA-camptothecin ternary complex models (16, 17) and structure-activity relationships studies (18), there is a space for substitutions at positions 7 and 10 on the B and A ring of the quinoline moiety of CPT, respectively, without decrease in activity. Therefore, we synthesized conjugates of the 16- and 18-mer TFO linked to 7-(2-aminoethyl)camptothecin via an hexyl linker (structure in Fig. 2). The DNA recognition and cleaving properties of these conjugates were compared with the ones of the 10-carboxycamptothecin analogs. The differences in intensity of cleavage observed for the four different combinations of TFO16/18 and 7/10CPT provide some structural infor-

**FIG. 5.** Effect of the spacer length together with the length of the triplex helix. Sequence analysis of the topoisomerase I-mediated cleavage products on the 324-bp target duplex (50 nM) is shown. The details are as in Fig. 3. Duplex alone (lane 1) or incubated with topoisomerase I (lane 2) and in the presence of 5 μM 10CPT (lane 3), 5 μM TFO16-L4–10CPT (lane 4), 5 μM TFO18-L6–10CPT (lane 5), 5 μM TFO18-L4–10CPT (lane 6), 5 μM TFO18-L6–10CPT (lane 7), 5 μM TFO20-L4–10CPT (lane 8), or 5 μM TFO20-L6–10CPT (lane 9) is shown.

**FIG. 6.** Comparison of the 7-(2-aminoethyl)camptothecin conjugates with respect to the 10-carboxycamptothecin conjugates. Sequence analysis of the topoisomerase I-mediated cleavage products on the 324-bp target duplex (50 nM) is shown. The details are as in Fig. 3. Target duplex (lane 1) incubated with topoisomerase I (lane 2) and in the presence of 5 μM 7CPT (lane 3), 5 μM TFO16-L6–7CPT (lane 4), 5 μM TFO18-L6–7CPT (lane 5), 5 μM TFO18-L6–10CPT (lane 6), 5 μM TFO16-L6–10CPT (lane 7), or 5 μM TFO18-L6–10CPT (lane 8).
mation regarding the ternary DNA-topoisomerase-poison complex.

Fig. 6 shows that the topoisomerase I cleavage profile of the free 7CPT differs markedly from the 10CPT one. Cleavage at site $b_1$ was stronger than at site $b_2$; the opposite effect was observed with 10CPT (compare lanes 3 and 6). Furthermore, cleavage at site $a$ and $f$ was weaker with 7CPT than with 10CPT. Noteworthy, the two TFO-7CPT conjugates induced cleavage only at sites $b_1$ and $b_2$ on the 3'-side of the triple helix. Cleavage at all the other sites of the drug ($a$ and $c-f$) was abolished, attesting that topoisomerase I-mediated DNA cleavage had been specifically directed by triplex formation. The 16-mer conjugate of 7CPT (TFO16-L6–7CPT, lane 4) specifically enhanced cleavage at site $b_1$ (7.6-fold) and reduced it at
Site b2 as compared with free 7CPT. The reverse profile was observed with the 18-mer conjugate TFO18-L6–7CPT (lane 5), which increased cleavage at site b2 by 10-fold and decreased it at site b1. On the contrary, 10CPT conjugates (TFO16-L6–10CPT and TFO18-L6–10CPT, lanes 7 and 8, respectively) stimulated cleavage preferentially at site b1 and only the longer, the 18-mer (lane 8), cleaved at site b2 to give a profile similar to the one observed with the 16-mer 7CPT conjugate (lane 4).

Concentration Dependence of Topoisomerase I-mediated DNA Cleavage—Next we compared the concentration dependence of the cleavage efficacy of the free drug to that of the TFO-drug conjugates. Fig. 7A shows topoisomerase I-mediated DNA cleavage in the presence of increasing concentrations of 10CPT (lanes 3–8) and TFO16-L4–10CPT (lanes 9–14) (from 0.01 to 5 μM). It is clear that the conjugate induces efficient DNA cleavage even at a concentration as low as 0.01 μM. At this concentration 10CPT alone (lane 3) shows little effect compared with topoisomerase I alone (lane 2). Band intensities were quantified by PhosphorImager analysis. Fig. 7B compares the percentage of cleavage as a function of the drug concentration for 10CPT (circles) and TFO16-L4–10CPT (triangles) at sites d, b2, b1, and a. TFO16-L4–10CPT induces cleavage at site b1 even at 0.01 μM; in contrast, at the other sites no cleavage was observed with the conjugate. Similar experiments were performed with various 16- and 18-mer conjugates of both 10CPT and 7CPT (Fig. 8). Here again the conjugates strongly enhanced topoisomerase I-mediated cleavage selectively at the triplex site b1/h2. The tethered molecules are considerably more potent than the free drug at inducing DNA cleavage.

Salt-induced Reversal of the Cleavage Complex in the Presence of Untethered and Tethered CPT—We then examined the reversibility of the topoisomerase I cleavage complexes induced by the drug alone and by the drug conjugated to the TFOs. CPT-stabilized cleavage complexes are rapidly reversible by increasing salt (NaCl) concentrations (19), and this method can be used to compare the stability of the cleavage complexes induced by different drugs. In Fig. 9, the effect of 1 μM 10-carboxycamptothecin is compared with that of conjugates TFO16-L4–10CPT and TFO16-L6–7CPT at 0.5 μM. For each sample, after topoisomerase I reaction, increasing concentrations of salt (0, 50, 100, 200, 400, and 600 mM NaCl) were added for 5 min. prior to proteinase K digestion. The results leave no doubt that the conjugates strongly increased the stability of the cleavage complex at site b1. High concentrations of NaCl were needed to reverse the cleavage complexes induced by the conjugated drug compared with the drug alone.

DISCUSSION

In the present study we probed the DNA-topoisomerase I cleavage complex targeted to a specific site using triple-helical structures of different lengths and conjugated to two camptothecin derivatives via spacers of variable size (Fig. 2). We first investigated how the length of the triple-helical structure at its 3′-end affected DNA cleavage by topoisomerase I at sites adjacent to the triplex site. As the length of the triplex increased at the 3′-end (TFO16 → TFO18 → TFO20) (Fig. 3), topoisomerase I cleaved further away from the triplex end, and a weak new site was observed at 7 bp from the 3′-end of the 20-mer (site h3).

The TFOs were linked either to 10CPT or 7CPT using for the former spacers of different length (butyl-, hexyl-, and decyldiamines) between the oligonucleotide and the drug, and for the latter γ-aminoacryloyl acid (Fig. 2). The capacity of the various conjugates to induce topoisomerase I-mediated DNA cleavage selectively at the triplex site was analyzed. As summarized in Fig. 10, all TFO conjugates were able in vitro to selectively direct the action of the camptothecin derivative.
Triplex-directed DNA Cleavage by Topoisomerase I

Fig. 9. Reversal of cleavage complexes. Salt-induced reversal of the cleavage complexes induced in the presence of the untethered drug (10CPT) at 1 μM and in the presence of the drug conjugated either at position 10 (TFO16-L4-10CPT) or at position 7 (TFO16-L6-7CPT) at 0.5 μM is shown. After 30 min of incubation with topoisomerase I, increasing concentrations of NaCl (0, 50, 100, 200, 400, and 600 mM) were added to the samples for 5 min prior to addition of SDS and proteinase K as described under "Experimental Procedures." The target DNA was used as control alone (lone DNA). The other details are as in Fig. 3.

at the triplex site and thus induced sequence-specific DNA cleavage by topoisomerase I. Upon binding of the TFO-drug conjugate to its specific target, DNA cleavage is strongly enhanced at the triplex/duplex junction (sites b1 and b2), where the inhibitor is positioned, and abolished at other sites (sites a and c-f). The results are reminiscent of those recently obtained with TFOs conjugated to other noncamptothecin topoisomerase I poisons such as indolocarbazole and benzoquinoxaline derivatives (7, 14), but here with the CPT derivatives both the selectivity and efficacy of cleavage are considerably reinforced. These findings suggest that the TFO conjugate behaves as a topoisomerase I poison with a negatively charged tail, which, because of electrostatic repulsion, eliminates its binding to DNA or DNA-topoisomerase I sites except at the site where the TFO finds a target sequence. Binding of the TFO to this DNA site delivers the topoisomerase I poison selectively to the adjacent cleavage site in such a position to inhibit the religation of the topoisomerase I-induced DNA breaks. This strategy offers great promise to enhance the selectivity of antitumor drugs.

The orientation in which the drug is brought in the ternary complex upon triplex formation is an important feature for cleavage stimulation. Collectively, the data demonstrate that the elaboration of a conjugate molecule requires an optimization of the two tethered components but also a precise design of the linker chain to locate the poison in the ternary complex at its preferential site.

Structural data are available for the topoisomerase I-DNA complex (17, 20, 21), but thus far the exact positioning of camptothecin in the cleavage complex has not yet been determined. Camptothecin does not interact (or loosely) with either DNA alone or topoisomerase I alone but does within the cleavage complex (16, 17). Our set of TFO-CPT conjugates provide a useful molecular tool to probe the structure of the topoisomerase I-DNA-poison ternary complex. By using TFO and linkers of variable length and CPT derivatives differently attached to the TFO, we can change the orientation of the CPT moiety with respect to the DNA-topoisomerase I complex. The cleavage profile of TFO16-L6-7CPT resembles the one of TFO18-L6-10CPT with prominent cleavage at site b1 (Fig. 6), whereas the 18-mer conjugate of 7CPT (TFO18-L6-7CPT) enhanced cleavage at site b2, resembling more to the 20-mer 10CPT conjugates (Fig. 5). These two sites, b1 and b2, are located 4 and 7 bp away from the 3'-end of the triplex formed by the TFO16, respectively. With both 7CPT and 10CPT, we built a preliminary model for the ternary complex with the conjugate bound to its triplex site. In the case of the 7CPT conjugates and the potent TFO16-L4-10CPT, we found that the distance between the 3'-end of the TFO and the CPT moiety was clearly too short to enable the insertion of the CPT residue between the −1 and +1 bases of the b1 site. By molecular simulations, Fan et al. (16) suggested that the camptothecin is pseudo-intercalated between the bases −1 and +1 in the enzyme-DNA complex. A slightly different configuration has been proposed by Redinbo et al. (17) on the basis of the crystal structure of the DNA-topoisomerase I complex. They proposed that the camptothecin is inserted in the DNA in the space vacated by the base in +1, a guanine, that flips out of the DNA duplex. We tested both possibilities, but we were not able to explain our results with an intercalation model for CPT. There is some degree of flexibility in the topoisomerase I-DNA complex even at the active site (21), and therefore, other configurations might be possible. A complex where the camptothecin moiety is extended in the major groove and points toward the enzymatic active site could better fit with our findings. On the other hand, an intercalation model is still possible, if the presence of the triple helix or the enzyme distorts DNA (21). Different architectures for the topoisomerase I-DNA-CPT ternary complexes may coexist.

On the basis of gas phase computations, Kerrigan and Pilch (22) have recently proposed a model for CPT interaction with the topoisomerase I-DNA covalent binary complex. Their computations suggest that the A ring of CPT is directed toward the major groove, which would be fully consistent with the data obtained for the TFO conjugate in which the CPT is attached via the 10-position, because any putative structure that leads to site-specific cleavage must involve triplex formation in the major groove. In this context it is interesting to note that the TFO conjugates formed from 7CPT are no less effective in mediating topoisomerase I-dependent cleavage of the target duplex than those involving attachment via the 10-position of CPT, although some adjustments of the length of the tether between the oligonucleotide and CPT moieties are required, as noted above. If the tethered CPTs were associated with the bound topoisomerase I-DNA binary complex via an intercalative interaction, it would be necessary to posit either that the TFO-CPT conjugates attached through positions 7 and 10 had the CPT positioned in the same fashion, necessitating the inclusion of part of the oligomethylene tether within the intercalated complex or else that the nature of the forces that secure the intercalated CPT between DNA base pairs are such that...
alternative bound orientations may be readily accessible because of small energy differences between them. The latter possibility derives support from two recent publications (8, 9), which demonstrated that the attachment of CPT through position 10 (A ring) to minor groove-binding hairpin pyrrole-imidazole polyamides afforded a conjugate (minor groove binder-CPT) that resulted in topoisomerase I-mediated cleavage at the site of binding of the conjugate. Furthermore, a comparison between TFO-CPT and minor groove binder-CPT conjugates showed that the two class of conjugates are equal both in efficacy and in cleavage sites, despite the fact that the CPT moiety is brought from the major and minor groove side, respectively (8). The possibility that different derivatives of CPT may be capable of binding to the topoisomerase I-DNA covalent binary complex in distinctly different orientations has potentially important implications for the design of novel inhibitors of topoisomerase I function.

Lastly, it is important to emphasize that the TFO-CPT conjugates described here are extremely potent at inhibiting topoisomerase I. Their action is both sequence-specific and efficient. The positioning of the CPT moiety by triplex formation increases the local drug concentration at the targeted site, and DNA cleavage can be detected using nanomolar concentrations of the conjugate (Figs. 7 and 8). In terms of concentrations, this is a significant improvement compared with CPT alone. Moreover, the stability of the covalent topoisomerase I-DNA cleavage complex is strongly increased (Fig. 9), and this can be important for the use of topoisomerase I poisons as anticancer agents. In the future, triple helix-directed targeting of antitumor-active topoisomerase I poisons may be exploited further to improve the efficacy of chemotherapeutic cancer treatments by targeting strong and irreversible topoisomerase I-mediated DNA cleavage selectively at specific genes.

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Fig. 10. Comparison of the conjugates. The ratio between the intensity of cleavage in the presence of the conjugate and the intensity in the presence of the untethered corresponding camptothecin derivative is represented on a logarithmic scale at different sites along the DNA fragment used as a substrate for topoisomerase I cleavage (data from three different assays).
Design and Optimization of Camptothecin Conjugates of Triple Helix-forming Oligonucleotides for Sequence-specific DNA Cleavage by Topoisomerase I
Paola B. Arimondo, Alexandre Boutorine, Brigitte Baldeyrou, Christian Bailly, Masayasu Kuwahara, Sidney M. Hecht, Jian-Sheng Sun, Thérèse Garestier and Claude Hélène

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