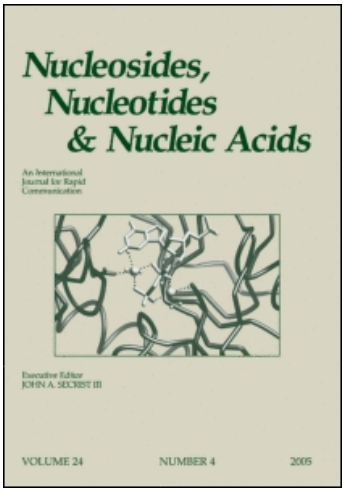

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Linkage of a Triple Helix-Forming Oligonucleotide to Amsacrine-4-carboxamide Derivatives Modulates the Sequence-Selectivity of Topoisomerase II-Mediated DNA Cleavage

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LINKAGE OF A TRIPLE HELIX-FORMING OLIGONUCLEOTIDE TO AMSACRINE-4-CARBOXAMIDE DERIVATIVES MODULATES THE SEQUENCE-SELECTIVITY OF TOPOISOMERASE II-MEDIATED DNA CLEAVAGE

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ABSTRACT: Amsacrine-4-carboxamide-oligonucleotide conjugates were synthesized and studied for their capacity to form DNA triple helices and to alter human topoisomerase II binding and cleavage properties. The intercalating agent was attached to the 3'- or the 5'-end of a 24 nt triple helix-forming oligonucleotide via linkers of different lengths. The stability of these DNA triple helices was investigated by gel retardation and melting temperature studies using a synthetic 70 bp DNA duplex target. The effect of the conjugates on DNA cleavage by topoisomerase II was evaluated using the 70 bp duplex and a 311 bp restriction fragment containing the same triple helix site. The conjugate with the amsacrine derivative linked to the 3' end of the TFO via a hexaethylene glycol linker modulates the extent of DNA cleavage by topoisomerase II at specific sites.

INTRODUCTION

Amsacrine (4'-(9-acridinylamino)-methanesulfon-*m*-anisidide or *m*AMSA[1], Figure 1) is an anilino-acridine antitumour drug mainly used for the treatment of leukemia in

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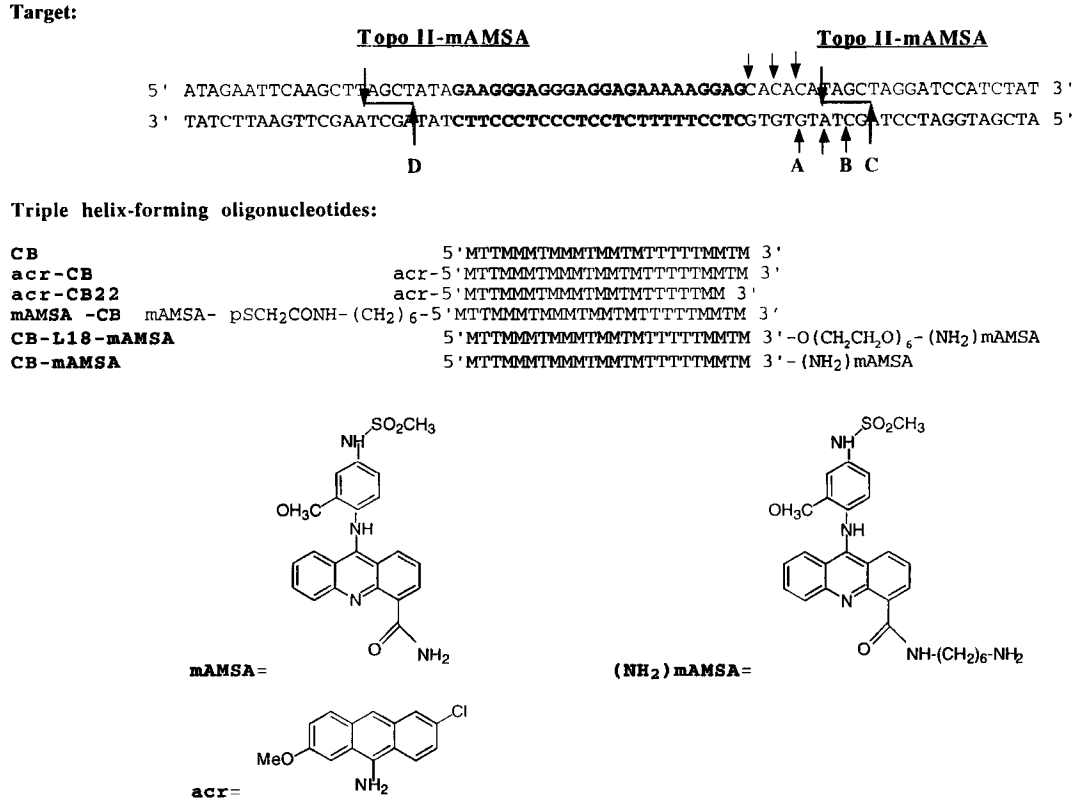


FIG 1. Sequence of the 70 bp target duplex and TFO conjugates. The sequence of the triple helix binding site is indicated with bold characters. M represents 5-methyl-2'-deoxycytosine. Topoisomerase II-mediated cleavage sites are indicated by arrows. The chemical structure of the drugs is represented.

children [1,2]. This drug, developped in the 1970s, intercalates into DNA and inhibits topoisomerase II [3]. This ubiquitous DNA relaxing enzyme which is essential for replication, transcription, genetic recombination and chromatin organization [4], is a privileged target for many antitumour and antiviral drugs [5]. Amsacrine functions as a conventional topoisomerase II poison (like etoposide), stabilizing the covalent 5'-phosphorotyrosyl DNA-topoisomerase II adducts, usually referred to as the cleavable complexes [6]. Under physiological conditions, the DNA cleavage and ligation reactions catalysed by the enzyme are tightly coordinated and the covalent intermediate is barely detectable. The cleavage is coupled with the religation step to restore continuity in the

DNA duplex. Amsacrine converts topoisomerase II into a cell poison by blocking the religation step, thereby enhancing the formation of persistent DNA breaks responsible for cell death [7]. Amsacrine preferentially traps a subset of topoisomerase II cutting sites, especially those containing an adenine immediately 3' to the cleaved bond [8,9]. The drug binds at the interface of the enzyme-DNA complex and interacts preferentially with $T\downarrow A^{+1}$ and $C\downarrow A^{+1}$ sequences (the arrow denotes the site of cleavage by the enzyme).

Recently, it has been shown that the sequence selectivity of topoisomerase I cleavage can be modulated by attachement of an inhibitor, such as camptothecin [10,11] or a glycosylated indolocarbazole derivative [11], to a triple helix-forming oligonucleotide (TFO). The same approach may be used to target DNA cleavage by topoisomerase II. Accordingly, we have attached amsacrine-4-carboxamide derivatives to a triple helix-forming oligonucloetide in order to direct DNA cleavage to a restricted number of T↓A or C↓A sites close to the oligonucleotide binding site on DNA. As a third strand, a 24 nt long oligonucleotide containing 5-methyl-2'-deoxycytidine residues (abbreviated M in the sequence on Figure 1) was chosen to reduce the pH dependence of its binding to the double-stranded DNA target. In this case, a DNA triple helix can be formed at pH 6.2 at room temperature. Amsacrine was attached to the 3'- or the 5'-end of the TFO via linkers of different chemical nature and length, as indicated on Figure 1. As a control, we also designed a conjugate containing an acridine-4-carboxamide attached to the 24 nt TFO or to a truncated oligonucleotide, 22 nt in length. Here we report the results of triplex formation and topoisomerase II-mediated DNA cleavage studies with the various conjugates.

MATERIALS AND METHODS

Chemicals and enzymes

Amsacrine and etoposide (Sigma) were dissolved in dimethylsulfoxide (DMSO) at 1 mM and then further diluted with water. Restriction endonucleases, shrimp alkaline phosphatase, proteinase K and T4 polynucleotide kinase were obtained from Boehringer Mannheim and used in the provided activity buffer. Deoxyribonuclease I (DNase I) was from Sigma. Human topoisomerase II was from Topogen Inc.. [γ–^{32}P]ATP was from Amersham. Oligonucleotides were purchased from Eurogentec and purified using sephadex G-25 quick spin columns (Bio-Rad).

Synthesis of oligonucleotide-drug conjugates

The synthesis of the amsacrine-4-carboxamide derivative has been previously reported [12]. The amino group of the aliphatic chain of amsacrine-4-carboxamide was linked to the phosphorylated 5'- or 3'-end of the TFO, or to the phosphorylated hexaethylene glycol linker arm at the 3' end of the TFO, using a procedure previously described [13,14]. Briefly, 150 μg of 3' phosphorylated oligonucleotide were first precipitated as hexadecyltrimethylammonium salt and then dissolved in 50 μl of dry DMSO. *N*-methylimidazole (5 μl), dipyridyl disulfide (25 μl) and triphenylphosphine (25 μl) (each 1.2 M solutions in DMSO) were then added to the mixture. After 20 min incubation at room temperature, triethylamine (5 μl) and the drug (20 μl, 30 mM in DMSO) were added. The mixture was stirred for 30 min prior to precipitation of the oligonucleotide with $LiClO_4$ and purification by reverse phase HPLC using a linear acetonitrile gradient (5-40% CH_3CN in 0.2M $(NH_4)OAc$). The average yield was 60%.

DNA purification and radiolabelling

Synthetic single-stranded oligonucleotides (10 pmol) were 5'-labelled with [γ–^{32}P]ATP using T4 polynucleotides kinase. Unincorporated radioactive ATP was eliminated by centrifugation through sephadex G-25 quick spin columns. The digestion of the plasmid pNG1 with *Pvu* II and *Eco*RI yielded a 311-mer fragment suitable for 5'-end labelling with T4 polynucleotide kinase. The detailed procedures for isolation, purification and labelling of such a duplex DNA fragment have been described elsewhere [15]. After electrophoresis on a non-denaturing 6% polyacrylamide gel, the 5'-end labelled product was cut out of the gel and eluted overnight in 10 mM ammonium acetate, 10 mM magnesium acetate. The purified DNA was then precipitated twice with 70% ethanol prior to resuspension in 10 mM Tris buffer, pH 7.0, containing 10 mM NaCl.

Gel retardation and DNase I footprinting assays

For DNA triplex gel retardation assays, one strand of the duplex (20 nM) was 5' end-labelled with [γ–^{32}P]ATP in the presence of T4 polynucleotide kinase, to which were added increasing concentrations (20 nM-100 μM) of the TFOs in 50 mM MES pH 6.2, 120 mM KCl and 10 mM $MgCl_2$ and in the presence of 10% sucrose and 0.5 μg/μl

tRNA. Samples were incubated overnight at RT prior to electrophoresis on a non-denaturing 12% polyacrylamide gel. Apparent dissociation constants for triplex formation, K_d, were calculated according to a two-state model [16]. DNase I footprinting experiments were performed as previously described [17].

Melting temperatures

A Kontron Uvikon 940 spectrophotometer with 1 cm optical pathlength quartz cuvettes was used to study thermal denaturation and renaturation of the triplex. The cell holder was thermoregulated by an 80/20% water/ethylene glycol circulating mixture. Sample temperature was decreased from 90 to 0°C and increased back to 90°C at a rate of 0.2°C/min with absorption readings at 260 and 405 nm taken every 1-1.2 °C. Samples were maintained at each extreme temperature for an additional 10 min. For the melting temperature (Tm) analysis, absorption at 405 nm was subtracted from that at 260 nm and plotted against temperature (°C). Tm values were determined from first derivative plots.

Topoisomerase II cleavage assays

The radiolabelled DNA duplex (50 nM) was incubated for 1h at room temperature with the TFO, with or without the test drug, in a 50 mM Tris-HCl buffer pH 6.2 containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 μg BSA (total volume 20 μl). Topoisomerase II (10 units) was then added to each sample for 1h at room temperature. DNA-topoisomerase II complexes were dissociated by addition of SDS (0.5%) and proteinase K (500 μg/ml) followed by heating at 55°C for 35 min. After ethanol precipitation, the samples were resuspended in 6 μl of formamide dye, heated at 90°C for 4 min and then chilled on ice for 4 min, prior to electrophoresis on a denaturing (8 or 15% as indicated) polyacrylamide gel containing 7.5 M urea in 1xTBE buffer. A Molecular Dynamics 445SI Phosphorimager was used to quantitate band intensities.

RESULTS

Topoisomerase II cleavage of the 70 bp fragment in the presence of the conjugate mAMSA-CB

Initially, we synthesized the conjugate mAMSA-CB where the intercalator is attached to the 5'-end of the 24 nt TFO CB (Figure 1). A synthetic 70 bp fragment

containing the triplex binding site was used to evaluate the effect of the conjugate on topoisomerase II cleavage. It has been previously shown that triplex formation at more than 3 bp from the cleavage site does not interfere with activity of topoisomerase II [18]. The target duplex, labelled either on the oligopurine (R) or on the complementary oligopyrimidine (Y) strand, was incubated with topoisomerase II in the presence of 2 μM of the conjugate or the TFO alone or the TFO plus the unlinked drug. Amsacrine was also used as a control. The target sequence was designed in order to contain (Figure 1): (i) two Topo II cleavage sites, corresponding to mAMSA specific sites, T↓AGCT↑A [8], situated on both sides of the triple helix; and (ii) three strong Topo II cleavage sites, C↓AC↓AC↓A, on the 3'-side of the triplex [18]. A typical sequencing gel experiment obtained with the target duplex radiolabelled on the Y strand is presented in Figure 2. Under the experimental conditions used (pH 6.2, 120 mM KCl and 10 mM $MgCl_2$ at room temperature), the triple helix can be easily formed (Table 1) and topoisomerase II retains its DNA cleavage activity.

Three topoisomerase II cleavage sites, marked A, B and C, can be seen on the 3' side of the triplex. Sites A and B correspond to the first and the last of the three designed sites C↓AC↓AC↓A, on the 3'-side of the triplex. Site C is the specific mAMSA site T↓AGCT↑A inserted at the 3' end of the triple helix site. A fourth site D may be detected on the 5' side of the triplex, corresponding to the other T↓AGCT↑A site. This later site was easier to detect using the target duplex labelled on the oligopurine strand. The presence of the triple helix decreased markedly the cleavage at site A close to the 3' end of the triplex (1 bp) whereas the cleavage at site B, further away from the triplex/duplex junction (5 bp), was not significantly affected. Cleavage at site A was restored upon addition of a 10-fold excess of amsacrine compared to the oligonucleotide. Interestingly, we noted that the addition of the conjugate mAMSA-CB to the duplex resulted in a complete loss of cleavage at site A but a major increase of topoisomerase II cleavage at site C, which is located at about one helix turn from the 3'-end of the triplex site. Cleavage at this consensus site T↓AGCT↑A was 6 times more intense with the conjugate compared to free mAMSA. The same results were obtained when the target was labelled on the complementary R strand (data not shown), in agreement with the 4 bp staggered strand breaks by topoisomerase II.

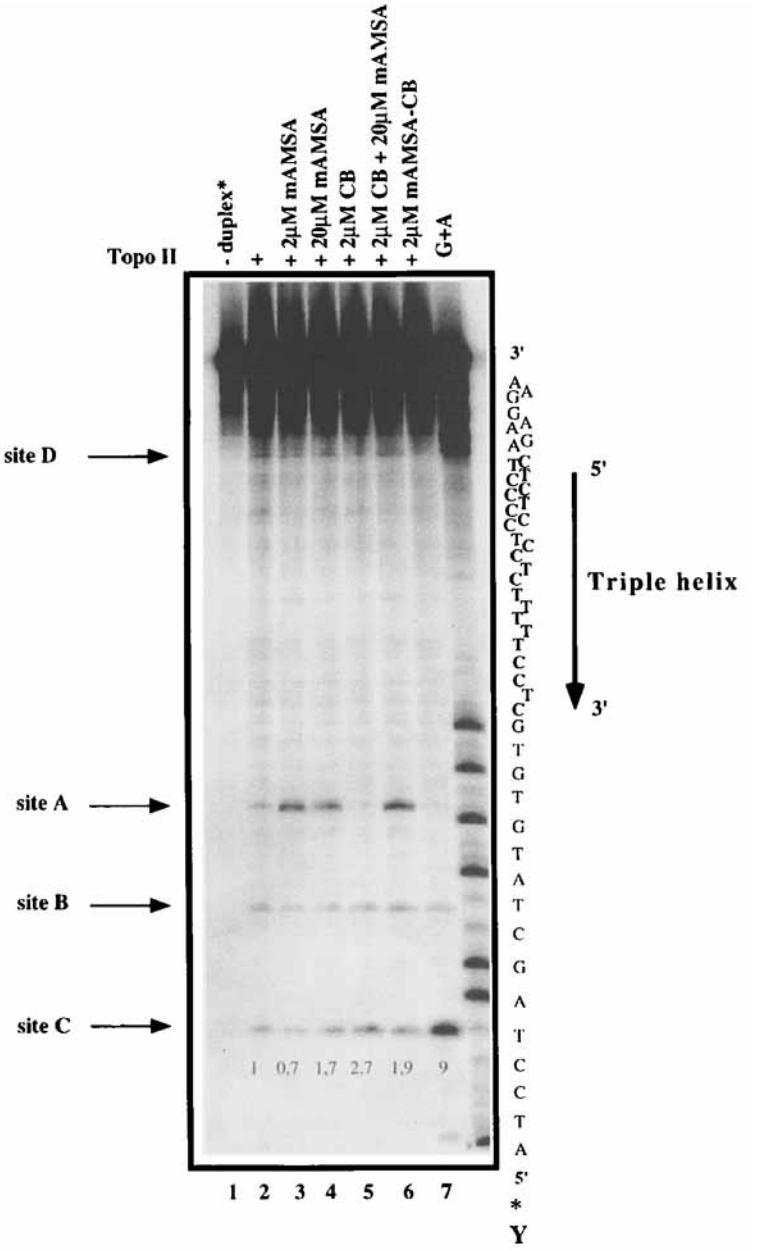


FIG 2. Effects of the TFO-drug conjugates on the cleavage of the 70 bp duplex by topoisomerase II. The duplex radiolabelled on the pyrimidine-rich strand (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 2-20 μM amsacrine (lanes 3 and 4), 2 μM oligonucleotide CB (lane 5), 2 μM oligonucleotide CB and 20 μM amsacrine (lane 6) or 2 μM CB-mAMSA conjugate (lane 7). The track labelled G+A represents formic acid-piperidine markers specific for purine residues. The position of the topoisomerase II cleavage sites is marked by arrows from A to D.

TABLE 1: Binding and cleavage properties of the conjugates

	Tm (°C)	Kd (μM)	IC
mAMSA	-	-	1.7
CB	39	0.8	2.7
acr-CB	41	0.7	2.9
acr-CB22	43	0.9	2.7
mAMSA-CB	49	0.5	9.5
CB-L18-mAMSA	36	0.9	1.7
CB-mAMSA	nd	1.8	9.0

The index of cleavage (IC) refers to the cleavage enhancement measured at site C in the presence of the indicated molecule relative to the cleavage intensity measured with topoisomerase II alone.

The enhanced cleavage at site C cannot be attributed to a specific recruitment of topoisomerase II by the conjugate mAMSA-CB, because upon triplex formation the inhibitor is positioned at the 5' duplex/triplex junction, whereas the cleavage is detected on the other side, at the 3'-end of the triplex. We may attribute the enhanced cleavage as the result of a triplex-mediated conformational change of the duplex that potentiates access and DNA cleavage by topoisomerase II.

These preliminary results prompted us to synthesize new conjugates with the amsacrine derivative linked to the 3' end of the TFO. We made the hybrid CB-mAMSA and a related compound bearing a much longer linker arm between the drug and the TFO, in order to enable the intercalator to reach site C. A hexaethylene glycol linker (L18) was chosen on the basis of molecular modeling study (CB-L18-mAMSA).

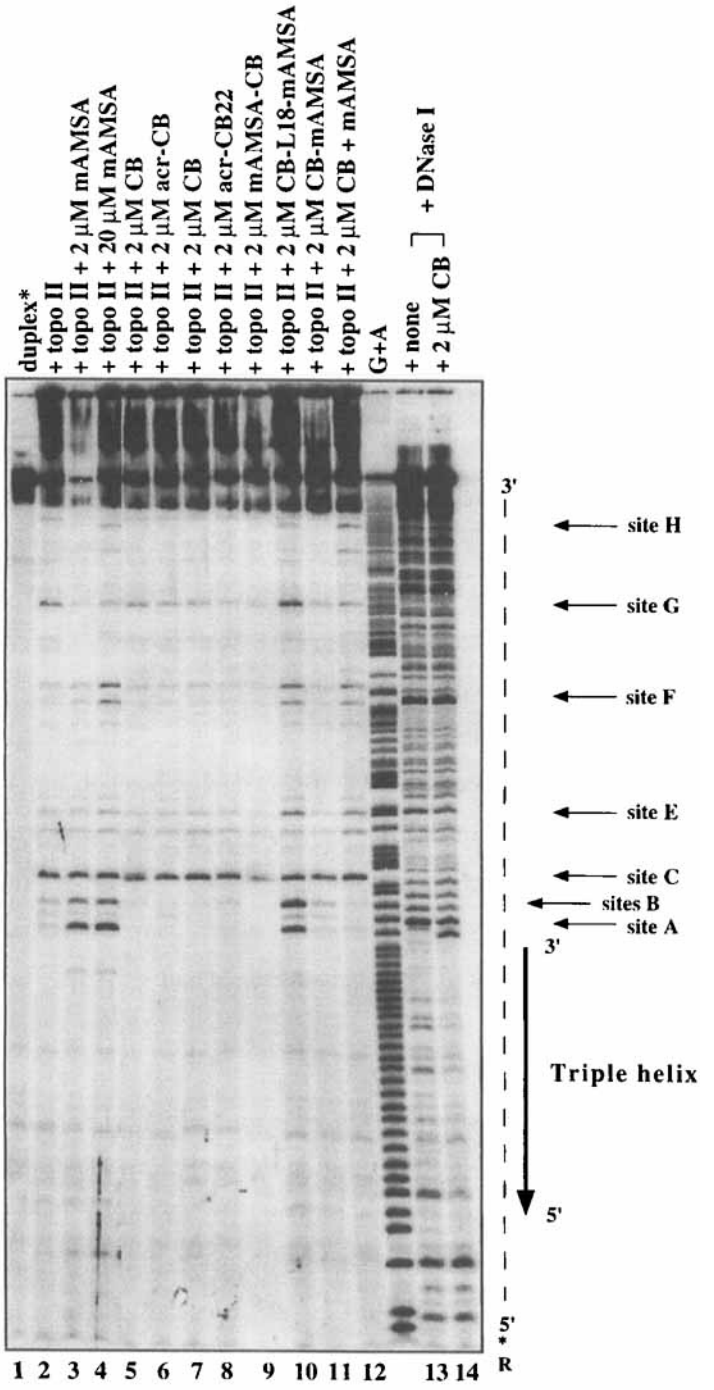
Because topoisomerase II recognition and cleavage is affected by the conformational changes induced by triplex formation, we first studied the ability of these conjugates to form a triple helix on the target duplex.

Stability of the DNA triple helices

Two complementary methods, thermal denaturation and electrophoresis mobility shift assay, were used to evaluate the stability of the triple helices and the effects of the various drugs attached to either the 3'-end or the 5'-end of the TFO. In both cases, the triplex was formed at pH 6.2 and at room temperature in a buffer containing 120 mM KCl and 10 mM $MgCl_2$. The melting temperature of the triplex was determined in the presence of the TFO alone or after addition of 1 μM amsacrine and compared with the Tm values measured with the various conjugates. From the electrophoresis mobility shift assays, we calculated the apparent dissociation constants of triplex formation. The Tm and K_d values are summarized in Table 1. There is a good agreement between the results obtained by the two methods. The triplex stability does not vary greatly. A stabilization of the triplex structure was obtained when the amsacrine derivative was tethered to the 5' end of the TFO (mAMSA-CB). In this case, the Tm value was increased by 10°C and the Kd was significantly decreased. In contrast, linkage of the amsacrine derivative to the 3'-end of the TFO decreased triplex stability, but the destabilizing effect was less pronounced with the conjugate containing a long linker arm (CB-L18-mAMSA compared to CB-mAMSA). Previous studies have demonstrated that the stabilizing effect of an intercalating drug is more important at the 5' triplex/duplex junction than on the 3' side [19,20]. With the two acridine conjugates, the stabilizing effect was weak. The overall weak stabilizing effect of the various conjugates probably results from the protonated cytosine residues of the terminal C•G*C^+ base triplets, which may be responsible for an electrostatic repulsion with the positively charged intercalator.

Topoisomerase II-mediated DNA cleavage

A first series of experiments was performed with the synthetic 70 bp duplex target as described above. The extent of cleavage at site C with each molecule is reported in Table 1. The cleavage index refers to the cleavage enhancement measured with the indicated molecule relative to the cleavage intensity measured with the topoisomerase II alone without added ligand or third strand oligonucleotide. The highest cleavage enhancement was observed with the amsacrine derivative connected to the TFO via a short linker. There was no difference between the drug attached to the 3' and to the 5' end of the TFO. Moreover, the cleavage enhancement was weak with the hybrid CB-L18-



mAMSA containing a longer optimized linker between the two moieties. In this case, cleavage at site C was not more pronounced than with amsacrine alone or with the two acridine-TFO conjugates. No direct correlation could be deduced between the extent of cleavage at site C and the degree of stability of the triplex, inferred from the Tm and electrophoresis mobility shift assays.

Next, we cloned the oligopurine•oligopyrimidine binding site into a plasmid vector and a 311 bp fragment was prepared with the radiolabel attached to the 5'-end of the R strand. A sequencing gel showing topoisomerase II-mediated cleavage on the 311 bp DNA target in the presence of various conjugates and drugs is presented on Figure 3. Prior to the description of the cleavage sites, it is important to refer to the footprinting experiments with the TFO. In the presence of the TFO, the cutting of the duplex target by DNase I was markedly reduced within the triple helix site and a characteristic enhancement of DNase I cleavage was observed at the 3'-triplex/duplex junction (lanes 13 and 14). Such a footprinting profile attests that under the experimental conditions used for the topoisomerase II experiments the triple helix was formed.

With the longer target, 311 bp, the cleavage by topoisomerase II at site C was not considerably increased, in contrast to what was observed with the shorter target, 70 bp in length. However, it is still clear that cleavage at sites A and B remains inhibited in the presence of the TFO-amsacrine conjugates (3' or 5' linked, lanes 9 and 11) as well as with

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FIG 3. Effects of the TFO-drug conjugates on the cleavage of the 324 bp fragment by topoisomerase II. The DNA radiolabelled on the pyrimidine-rich strand (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of amsacrine (2 and 20 μM, lanes 3 and 4), oligonucleotide CB (2 μM, lanes 5 and 7), the different TFO-drug conjugates (2 μM, lanes 6-11) or the combination of oligonucleotide CB and amsacrine (2 μM lane 12). The track labelled G+A represents formic acid-piperidine markers specific for purine residues. The last two lanes refer to the DNase I footprinting experiments performed with DNA alone (lane 13) or the DNA plus the oligonucleotide CB (2 μM, lane 14) to identify the position of the triple helix binding site. The position of the topoisomerase II cleavage sites is marked from A to H.

the acridine-TFO hybrids (lanes 6 and 8) or the TFO alone (lanes 5 and 7). Cleavage at these two sites, both C↓A sites, was observed with topoisomerase II alone (lane 2), with amsacrine (lanes 3 and 4) and also with the conjugate CB-L18-mAMSA (lane 10). The pattern of cleavage obtained with this latter conjugate was quite different from that observed with the other conjugates. A densitometric analysis of the cleavage sites produced with CB-L18-mAMSA revealed interesting features. The cutting profile is reminiscent of that of amsacrine alone but the band intensities are different. For example, cutting at site B is 3 times more pronounced with CB-L18-mAMSA than with amsacrine, as is the case at site E, located at 22 nucleotides from the 3'-end of the triplex, and as site G. On the other hand, cleavage at site H is reduced. With this 311 bp target, both conjugates CB-mAMSA (lane 11) and mAMSA-CB (lane 9) seem to abolish the stimulation of topoisomerase II cleavage observed with mAMSA. In contrast, the marked enhancement at site B in the presence of CB-L18-mAMSA may signify that the linker arm is well suited to allow the amsacrine moiety to intercalate near site B. The distance between the 3'-end of the triplex and site B corresponds ideally with the length of the linker inferred from the modeling analysis. It seems plausible that the stimulation of topoisomerase II cleavage at site B results from a specific recruitment of the enzyme by the conjugate CB-L18-mAMSA.

CONCLUSIONS

We have shown that a triple helix-forming oligonucleotide tethered to an amsacrine derivative modulates the extent of DNA cleavage by topoisomerase II. However the cleavage profiles are dependent upon the sequence and length of the target duplex, suggesting that the effect is regulated by the conformational properties of the DNA target. As previously shown with a TFO linked to topoisomerase I inhibitors [11], the DNA structure plays an important role in the recognition process between the enzyme and the drug-containing triplex system. Topoisomerase II is a homodimeric bulky enzyme, which can cover up to 28 bp upon interaction with duplex DNA [18]. It is therefore not surprising that the sequence context can be essential, especially in the presence of a triplex structure, which further increases the bulkiness of the molecular building. No direct correlation appeared between the effect of the conjugates on triplex stability and their

propensity to modulate the DNA cleavage by topoisomerase II. The study reported here, together with our related work with topoisomerase I poisons [11], suggest that the optimization of the linker arm between the drug and the TFO and the choice of potent inhibitors should be considered to target topoisomerases to specific sequences adjacent to a triple helix binding site.

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FOOTNOTES: [1] Abbreviations: bp, base pairs; mAMSA, amsacrine; Y, pyrimidine; R, purine; TFO, the triple helix-forming oligonucleotide; DMSO, dimethylsulfoxide; L18, hexaethyleneglycol.

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