Sequence-Specific Targeting of Duplex DNA Using a Camptothecin-Triple Helix Forming Oligonucleotide Conjugate and Topoisomerase I

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The sequence-specific targeting of duplex DNA using triple helix-forming oligonucleotides (TFOs) has attracted broad attention.[1] Currently, all such attempts to target DNA have used simple binding or the tethering of reactive chemical cross-linking agents.[2] An attractive option for biological applications would be the recruitment of a cellular enzyme which would be directed to perform a sequence-specific cleavage or modification. Such precedence is well-known in the sequence-specific targeting of RNA using the antisense approach.[3] The most advanced mechanism for antisense targeting is the recruitment of cellular RNase H for the cleavage of the RNA portion of a deoxyoligonucleotide-RNA hybrid.[4] No such cellular enzyme recruitment is currently known for duplex DNA targeting. We now report such a system using the enzyme Topoisomerase I (Topo I) and the Topo I inhibitor, camptothecin.

Topo I is a cellular enzyme present in high number in all mammalian cells.[5] Its function is to relax the superhelical twist in DNA which is generated during transcription and replication. The enzyme performs this function in a non-ATP dependent fashion, cleaving one strand of the DNA and forming a transient phosphodiester-tyrosine covalent linkage termed the cleavable complex.[5,6] This is followed by rotation about the cleaved position and religation of the DNA. Camptothecin and its analogs inhibit this process by binding to the covalent DNA enzyme complex and preventing religation.[5,6] We show that the trapping of the cleavable complex by camptothecin can be rendered sequence-specific by the appropriate tethering of a camptothecin analog to a TFO.

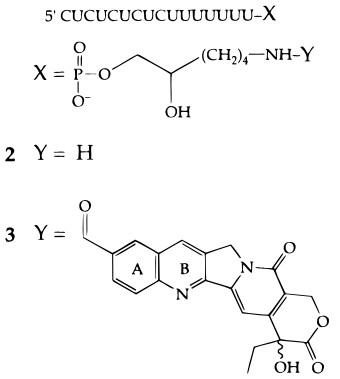

Figure 1. Sequence and structures: C = 5-methyl-2′-deoxycytidine, U = 5-propynyl-2′-deoxyuridine,[13] and all linkages are phosphodiester.

Camptothecin possesses significant antitumor activity, and consequently substantial effort has been expended in understanding the structural requirements for its activity.[7] Such work has elucidated that the A and B rings can be substituted in a variety of ways without the loss of activity. We chose the 10-carboxycamptothecin (**1**)[8] as a convenient analog for conjugation to a TFO. TFO **2** in Figure 1 was synthesized in standard fashion[9] from FMOC-protected C_6 amino link solid support[10] followed by NH_4OH deprotection. TFO **3** was produced by

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(10) This solid support was obtained from Clontech, Palo Alto, CA.

(11) MALDI-TOF MS: 5648.6 (5648.0 calc for M + H). The lactone moiety of camptothecin is known to equilibrate with the ring opened hydrolysis product under mildly basic pH and to recyclize to the lactone under mildly acidic conditions.[12] The MS data demonstrates that the conjugate is in predominately the active lactone form.

(12) Fassberg, J.; Stella, V. J. *J. Pharm. Sci.* **1992**, *81*, 676−684.

(13) This thymidine (T) analog was used because it had been previously shown to enhance triple helix formation relative to T.[14]

(14) Froehler, B. C.; Wadwani, S.; Terhorst, T. J.; Gerrard, S. R. *Tetrahedron Lett.* **1992**, *33*, 5307.

(15) The pTTL plasmid was created as follows: the Xho I-StuI fragment of pUHC13-3[16] was replaced with the following sequence containing the triple helix binding site, 5′-TCGAGTTTACCACTCCCTATCAGTGATAGAGAGAGAGAAAAAAAGAGAAGATCTGAGCTCGGTACCCGGGTCGACTAGGCGTGTACGGTGGGAGG-3′. To create the 3′-labeled restriction fragment in Figure 2, pTTL was digested with *BspH* I, labeled at the 3′ end, and digested with *Hind* III. To create the 3′-labeled restriction fragment in Figure 3, pTTL was digested with *Hind* III, labeled at the 3′ end, and digested with *BspH* I. In both cases, the ∼425 bp restriction fragment was isolated by nondenaturing gel electrophoresis.

(16) Gossen, M.; Bujard, H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5547−5551.

(17) The Topo I experiments were executed in total volume of 10 μL with a final concentration of each species as indicated. The ligands were added to solutions of radiolabeled restriction fragment (0.015−0.025 μg, 20−50 kcpm per reaction), Tris-HCl (50 mM, pH 7.2 at 37 °C), KCl (50 mM), $MgCl_2$ (10 mM), DTT (0.5 mM), EDTA (0.1 mM), and BSA (30 μg/mL). The DNA solutions were incubated with either 10-carboxycamptothecin **1** (30 μM, to solubilize the agent, 1% DMSO was present in the final reaction for this sample only), TFO **2**, or **3** (5 μM) prior to calf thymus Topo I addition (1 h, 37 °C). The Topo I reaction was initiated by the addition of enzyme (10 units) followed by incubation at 37 °C for 1 h. The reaction was quenched by adding 10% SDS (1 μL) and proteinase K (1.5 mg/mL, 1.2 μL), followed by incubation (50 °C, 30 min), and then addition of formamide dye, heat (95 °C), and PAGE (8%, denaturing). DNase I reactions were carried out under identical buffer conditions with the addition of enzyme (0.007 U per reaction, 1 min, 37 °C), and the reactions were quenched by the addition of EDTA (0.5 M, 1 μL).

(18) Strausfeld, U.; Richter, A. *Prep. Biochem.* **1989**, *19*, 37−48.

S0002-7863(97)00247-3 CCC: $14.00

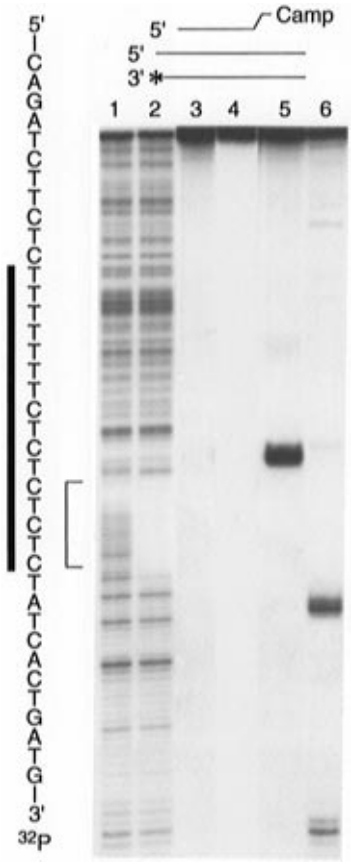

Figure 2. The trapping of cleavable complexes on duplex target 3′ end labeled on the bottom strand. A schematic of the reaction is shown at top of the figure. An asterisk (*) denotes the location of the ^{32}P radiolabel. The sequence of the targeted region is shown in the left margin. The black vertical bar highlights the TFO binding site on the sequence. The bracket marks the site of footprint protection: lane 1, target duplex incubated with DNase 1; lane 2, target hybridized to TFO **3** followed by DNase 1 treatment; lane 3, target incubated with Topo I; lane 4, target hybridized to TFO **2** followed by Topo I treatment; lane 5, target hybridized to TFO **3** followed by Topo I treatment; lane 6, target treated with free camptothecin **1** followed by Topo I treatment.

coupling the aliphatic amino group of **2** to a *N*-hydroxysuccinimide active ester of **1**. All products were purified by polyacrylamide gel electrophoresis (PAGE) and characterized by MALDI-TOF MS.[11]

The ability of the conjugate **3** to trap a cleavable complex was tested in a simple *in vitro* system. A restriction fragment (~1 nM) bearing the triple helix target site was 3′ end-labeled either in the bottom or topstand[15] and incubated[17] in the presence of TFO **3** (5 μM) and Topo I.[18] This was followed by protease treatment and denaturing PAGE analysis. In the case where the target was labeled on the 3′ end of the lower strand (Figure 2), a pronounced trapping (30% relative to unmodified target)[19] of the cleavable complex had occurred in a sequence-specific manner (Figure 2, lane 5). Underexposure of this lane demonstrated that the cleavage occurred at one site (data not shown).

This result contrasted the lack of targeting in the following control experiments shown in Figure 2. Topo I treatment alone with target produced no significant modification (lane 3). TFO **2** (5 μM) and Topo I with target produced no significant modification (lane 4). TFO **2** (5 μM), free 10-carboxycamptothecin **1** (5 μM), and Topo I with target produced no significant modification (data not shown). Topo I and free 10-carboxycamptothecin **1** at a high concentration (30 μM) without TFO generated cleavable complexes (lane 6) which were distinctly different than the TFO-directed result. At a lower concentration of **1** (5 μM) no pronounced cleavable complex was observed (data not shown). These controls demonstrate that the high efficiency, sequence-specific, Topo I-mediated targeting observed in lane 5 required the covalent linkage between the TFO and the camptothecin.

The footprinting experiment identified both the sites of triple helix binding and Topo 1 recruitment along the duplex DNA target. The target, 3′ end-labeled in the bottom strand, was treated for a limited time with DNase 1 in either the absence (Figure 2, lane 1) or presence of TFO **3** (lane 2). The footprint is visible in lane 2 and localized the TFO-directed Topo I recruitment observed in lane 5 to the predicted 5′ side of the triple helix binding site.[20]

The same experiments were performed with a target which was labeled at the 3′ end of the top strand. In this case, no significant TFO-directed cleavable complex is detected (Figure 3, Supporting Information). Thus the camptothecin conjugate **3** is specifically able to direct cleavable complex trapping on DNA with Topo I only to the bottom strand of the duplex DNA. This is the strand which is base paired to and opposite from the polypurine tract recognized in Hoogsteen binding fashion by the TFO.

The specificity and efficiency of this conjugate of 10-carboxycamptothecin **1** and a TFO are noteworthy. The conjugate is capable of targeting a Topo I lesion to a precise site based on triple helix recognition. It does so with high efficiency in an *in vitro* system. Such conjugates are worthy of further study in living cells for the sequence-specific targeting of genomic DNA via the recruitment of the cellular enzyme Topo I.

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Supporting Information Available: The experimental details are supplied for the preparation of the TFO camptothecin conjugate **3** and the autoradiogram and discussion of Figure 3 (3 pages). See any current masthead page for ordering and Internet access instructions.

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(19) This value is the average of three experiments (error ± 7%) and was obtained by densitometer analysis.

(20) The sequence in the cleaved region is shown in Figure 2 and does not contain a Topo I consensus cleavage sequence. There is only a weak consensus among camptothecin mediated calf thymus Topo I cleavage sites with the preference but not requirement being a G at the 5′ side and T at the 3′ side.[21]

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