Sequence-Specific Trapping of Topoisomerase I by DNA Binding Polyamide−Camptothecin Conjugates

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Abstract: Hairpin pyrrole−imidazole polyamides are synthetic ligands that bind in the minor groove of DNA with affinities and specificities comparable to those of DNA binding proteins. Three polyamide−camptothecin conjugates **1**−**3** with linkers varying in length between 7, 13, and 18 atoms were synthesized to trap the enzyme Topoisomerase I and induce cleavage at predetermined DNA sites. One of these, polyamide−camptothecin conjugate **3** at nanomolar concentration (50 nM) in the presence of Topo I (37 °C), induces DNA cleavage between three and four base pairs from the polyamide binding site in high yield (77%).

Introduction

Pyrrole−imidazole polyamides that target DNA sequences in the promoter have been shown to inhibit transcription of specific genes.[1−3] The question arises whether sequence-specific DNA binding polyamides could be designed to recruit endogenous cellular enzymes to modify DNA at specific gene sites reminiscent of RNase H cleavage of DNA·RNA hybrids in the antisense field.[4] Topoisomerase I (Topo I), an endogenous eukaryotic enzyme essential in replication and transcription, cleaves and religates the phosphodiester backbone of DNA in an ATP independent fashion.[5−7] The catalytic activity of Topo I involves a three-step process: (i) cleavage of one strand of DNA whereupon the enzyme is covalently linked to the 3′ phosphoryl end of the nick through a tyrosine residue termed the cleavable complex, (ii) passage of a segment of DNA through the nick, and (iii) resealing of the DNA break. This sequence is interrupted by the presence of the natural product camptothecin (Cpt), which stabilizes the cleavable complex and prevents religation of the DNA.[8] Camptothecin-stabilized Topo I−DNA adducts have been shown to cause premature termination of transcription elongation by RNA polymerase II.[9] A polyamide−camptothecin conjugate bound site specifically in the minor groove within a "coding region" of a specific gene might trap a covalent adduct of the Topo I enzyme with DNA. The site-specific protein−DNA adduct could be a gene-specific road block for the elongation of the RNA polymerase. Targeting Topoisomerase I to DNA by triple helix forming oligonucleotide conjugates has been previously reported by Matteucci[10] and Helene.[11] These oligonucleotide−camptothecin (and rebeccamycin) conjugates bind in the major groove of DNA. It would be interesting to compare major vs minor grove positioning of the Topo I poison. We report here our efforts to characterize the properties of a minor groove binding hairpin polyamide−camptothecin conjugate with regards to the structural requirements in the linker region for sequence specifically trapping the Topo I-mediated cleavable complex.[12]

Currently there are no three-dimensional structures of camptothecin bound to the DNA−Topo I cleavable complex although several groups have presented models.[6,13] Since the requirements for camptothecin presentation are unclear we designed and synthesized three polyamide conjugates **1**−**3** with linkers varying in length between 7, 13, and 18 atoms and chemical composition (alkyl chain vs hydroxy alkyl chain). For synthetic ease, camptothecin was attached to the hairpin turn. The eight-ring pyrrole−imidazole polyamide, ImPyPyPy-γ-PyPyPyPy-β-Dp (where Im is *N*-methylimidazole, Py is *N*-methylpyrrole, γ is γ-aminobutyric acid, β is β-alanine, and Dp is dimethylaminopropylamine), binds its match site 5′-AGTATT-3′ and its mismatch site 5′-AGTACT-3′ with equilibrium association constants of 3.5×10^9 and 5.0×10^8 M^{-1}, respectively.[14] We chose to modify camptothecin acid at C10, which has been shown by structure activity studies not to affect the ability of camptothecin to act as a Topo I poison.[15,16]

A plasmid pCW1 was constructed for the cleavage assay with polyamide−camptothecin conjugates **1**−**3** with one six-base pair

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match site 5′-AGTATT-3′ and two mismatch sites 5′-TGGAAA-3′ (single) and 5′-TGGACA-3′ (double) according to the pairing rules where Im/Py pairing binds G/C base pair and Py/Py are degenerate for A/T and T/A base pairs. The polyamide is oriented on the match site with the camptothecin to the 3′ side of the top strand. The sequences flanking the polyamide binding sites are 5′-GCCAAGTG-3′. Studies have shown that camptothecin-induced Topo I cleavage occurs preferentially but not exclusively between T and G.[17,18]

Results and Discussion

Synthesis of Polyamide−Camptothecin Conjugates 1−3. The polyamide conjugates **1**−**3** were synthesized in a stepwise manner from Boc-β-alanine Pam resin using Boc-chemistry protocols (Figure 3).[19] After the completion of the polyamide synthesis, the resin was split into three portions and three amino acid straight chain linkers were incorporated. The three polyamide conjugates were each cleaved by a single step aminolysis reaction with dimethylaminopropylamine and subsequently purified by reverse-phase HPLC chromatography. A camptothecin analogue **6** with a carboxy functionality suitable for conjugation was synthesized from 10-hydroxycamptothecin.[16a] Alkylation of 10-hydroxycamptothecin was accomplished with ethyl bromoacetate in refluxing acetone in the presence of anhydrous K_2CO_3 in 60% yield and the ester intermediate was hydrolyzed to form carboxymethyloxycamptothecin **6** in 90% yield. Activation of the camptothecin acid **6** was accomplished by dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). Polyamides with a primary amine on the turn when allowed to react with the activated acid in the presence of diisopropylethylamine (DIEA) afforded the polyamide−camptothecin conjugates **1**−**3**. The conjugates were purified by preparatory reverse-phase HPLC. Characterization and identity was confirmed by MALDI-TOF-MS.

Determination of Binding Affinities by Quantitative DNase I Footprinting Titration Experiments. Quantitative DNase I footprint titrations were performed on the 300 bp *Eco*RI/*Pvu*II restriction fragment of pCW1 to determine the equilibrium association constants of the polyamide−camptothecin conjugates **1**−**3** for two sites 5′-AGTATT-3′ and 5′-TGGAAA-3′ (Table 1). The 300 bp *Eco*RI/*Pvu*II restriction fragment includes the 115 bp *Eco*RI/*Afl*II restriction fragment used for the Topo I cleavage experiment. For all three conjugates **1**−**3**, the match site 5′-AGTATT-3′ was preferred over the single and double mismatch sites although we note the specificity of the conjugate overall appears compromised relative to the parent. The three conjugates show lower affinity for DNA than parent hairpin polyamide by a factor of 10, which is attributed to the attachment of the linker region at the γ turn.[20]

Polyamide-Camptothecin Conjugate Recruits Topo I to DNA and Cleaves DNA in High Yield. The ability of the polyamide−camptothecin conjugates to recruit Topo I and cleave DNA sequence specifically was tested by a simple in vitro system.[10] A radio-labeled DNA restriction fragment generated from plasmid pCW1 was 3′ labeled either on the top or bottom strand. The ligands were incubated with the DNA restriction fragment followed by addition of 10 units of calf thymus Topo I for 1 h at 37 °C. Following protein denaturation by treatment with SDS and protease workup, the DNA restriction fragments were analyzed by denaturing polyacrylamide gel electrophoresis. Analysis was first done on the bottom strand. Conjugate **3** at 50 nM concentration in the presence of 10 units of Topo I produces a distinct cleavage site between T∧G located 3−4 base pairs to the 3′ side of the polyamide binding site 5′-AGTATT-3′ (Figure 5A, lane 4). Weaker cleavage proximal to the mismatch site 5′-TGGAAA-3′ was also observed. Quantitation revealed a cleavage yield of 77% proximal to the match site 5′-AGTATT-3′ and 16% proximal to the single base pair mismatch 5′-TGGAAA-3′. Remarkably only 7% of the DNA fragment is left intact. In control experiments with 50 nM polyamide **4** alone or 50 nM camptothecin acid **6** alone, no cleavage was observed. Indeed concentrations of 1 μM were needed to observe cleavage with camptothecin acid **6** and three sites were observed at the bottom strand (Figure 5A, lane 9).

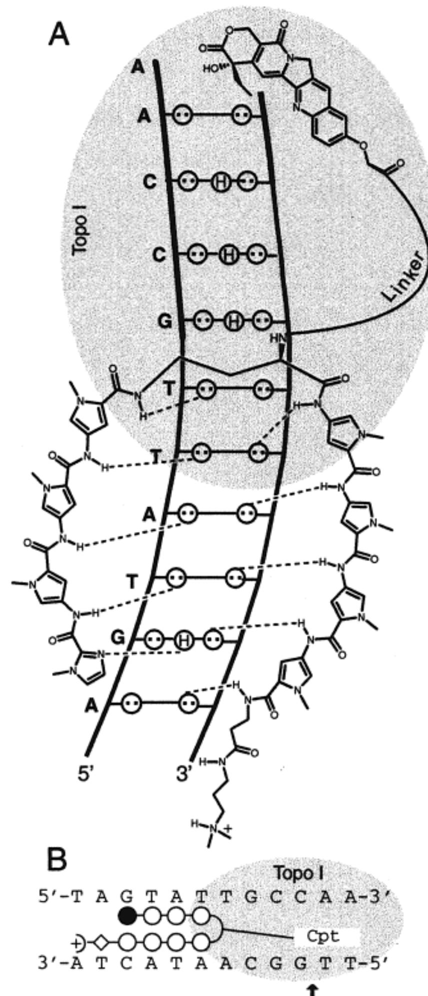

Figure 1. (A) Hydrogen-bonding model of a polyamide−camptothecin conjugate with a generic linker bound to the minor groove of 5′-AGTATT-3′ and Topo I. Circles with two dots represent the lone pairs of N3 purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogens of guanines. Putative hydrogen bonds are illustrated by dotted lines. (B) Binding model of a polyamide−camptothecin conjugate. Shaded and nonshaded circles denote imidazole (Im) and pyrrole (Py) rings, respectively. Diamonds, small ovals, and big ovals represent β-alanine (β), camptothecin (Cpt), and Topoisomerase I (Topo I), respectively. (*R*)-2,4-Diaminobutyric acid (γ) and dimethylaminopropylamine (Dp) are depicted as a curved line and a plus sign, respectively.

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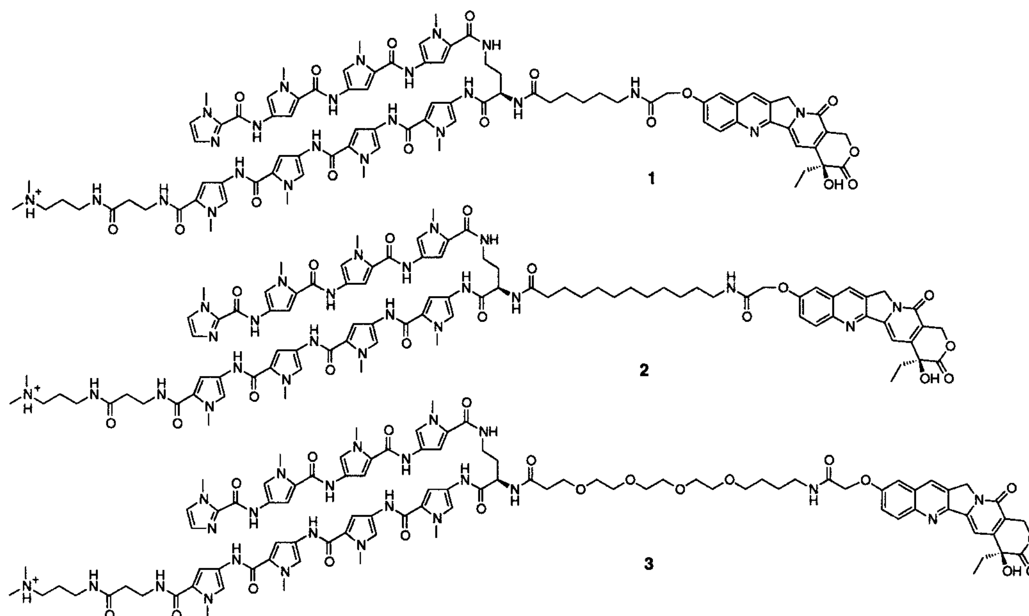

Figure 2. Structures of the hairpin polyamide−camptothecin conjugates: PA-CA5-Cpt (**1**), PA-CA11-Cpt (**2**), and PA-EG4-Cpt (**3**).

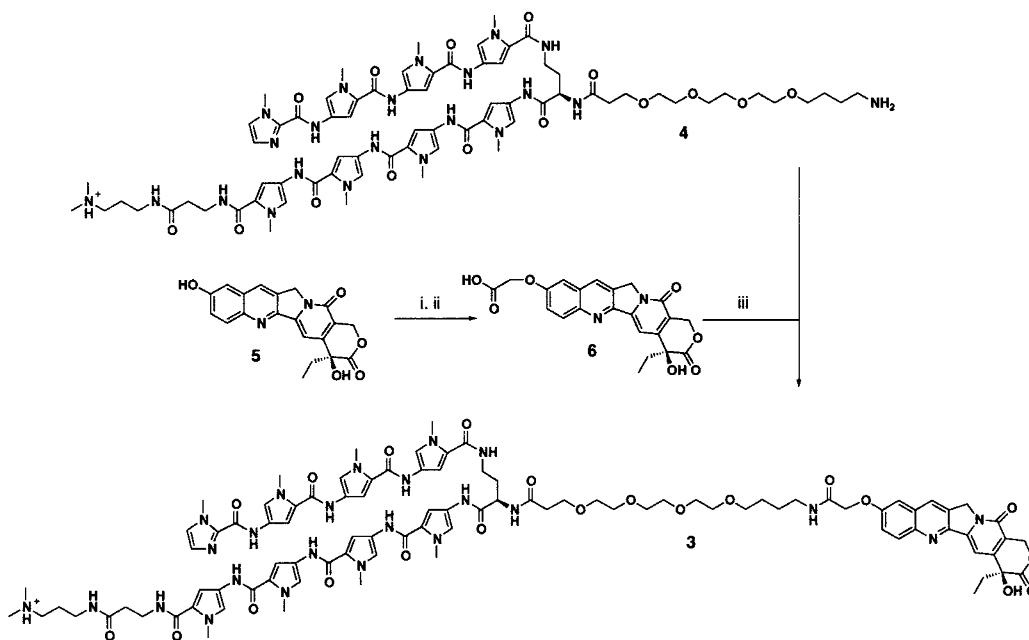

Figure 3. Synthetic scheme for polyamide−camptothecin conjugate **3**: (i) ethylbromoacetate, K_2CO_3 60%; (ii) EtOH, H_2O, K_2CO_3, 90%; (iii) camptothecin acid **6**, DCC, HOBt, 24% recovery.

Table 1. Equilibrium Association Constants (M^{-1}) for Polyamide−Camptothecin Conjugates

polyamide conjugate	5′-AGTATT-3′	5′-TGGAAA-3′
1	3.2×10^8 (0.1)	1.1×10^8 (0.2)
2	2.5×10^8 (1.0)	1.3×10^8 (0.3)
3	1.1×10^8 (0.3)	2.6×10^7 (1.0)

Moreover addition of 50 nM polyamide **4** and 50 nM camptothecin **6** with Topo I does not induce a cleavage band. Therefore a covalent linkage between the polyamide and camptothecin is required for efficient site-specific cleavage. Finally incubation of the conjugate **3** without Topo I revealed no cleavage, indicating that Topo I is necessary for the DNA cleavage.

Identical assays were then conducted on the top strand (Figure 5B). No cleavage was observed when 50 nM of conjugate **3** was incubated with 10 units of Topo I, indicating that the cleavage occurs only in one strand (Figure 5B, lane 4). Camptothecin acid **6** at 1 μM created four distinct cleavage sites (Figure 5B, lane 9). However, the cleavage yield was very low as demonstrated by the large amount of intact DNA. The strand-specific result leads us to conclude that the Topo I enzyme is oriented in one direction in the DNA/TopoI/polyamide−camptothecin complex. When 50 nM polyamide **4** and 1 μM camptothecin acid **6** were incubated together one of the four cleavage sites disappeared (Figure 5B, lane 8). Analysis shows that the cleavage site is also the polyamide binding site, suggesting that minor groove hairpin polyamide binding interferes sterically with the cleavage reaction.

Effects of the Linker Region on DNA Cleavage Yield. A series of polyamide−camptothecin conjugates differing only in the linker region were tested using the same in vitro assay

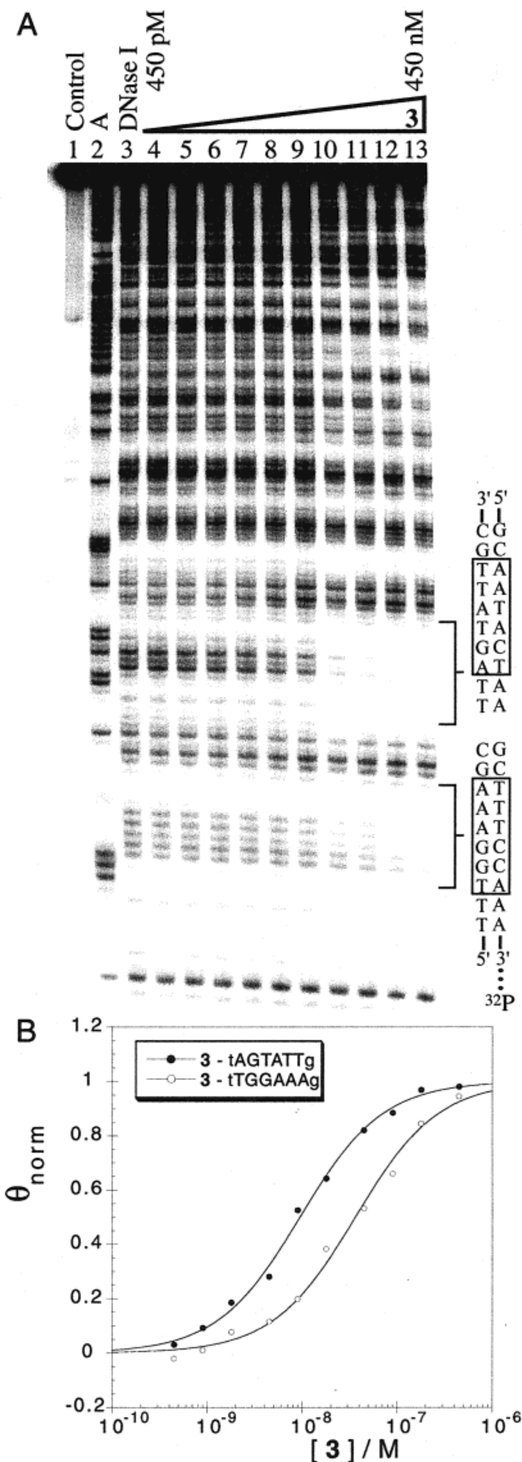

Figure 4. (A) Quantitative DNase I footprint titration experiment with PA-EG4-Cpt **3** on the 300 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pCW1: lane 1, intact DNA; lane 2, A specific reaction; lane 3, DNase I standard; lanes 4−13, 450 pM, 900 pM, 1.8 nM, 4.5 nM, 9 nM, 18 nM, 45 nM, 90 nM, 180 nM, 450 nM PA-EG4-Cpt **3**; the 5′-AGTATT-3′ and 5′-TGGAAA-3′ sites that were analyzed are shown on the right side of the gel. (B) Data from quantitative DNase I footprint titration experiments for PA-EG4-Cpt **3** binding to the two sites 5′-AGTATT-3′ and 5′-TGGAAA-3′. θ_{norm} points were obtained using storage phosphor autoradiography and processed by standard methods.[14] The data for the binding of conjugate **3** to 5′-AGTATT-3′ are indicated by filled circles and binding to 5′-TGGAAA-3′ by open circles. The solid curves are best-fit Langmuir binding titration isotherms obtained from the nonlinear least-squares algorithm where $n = 1$ as previously described.[14]

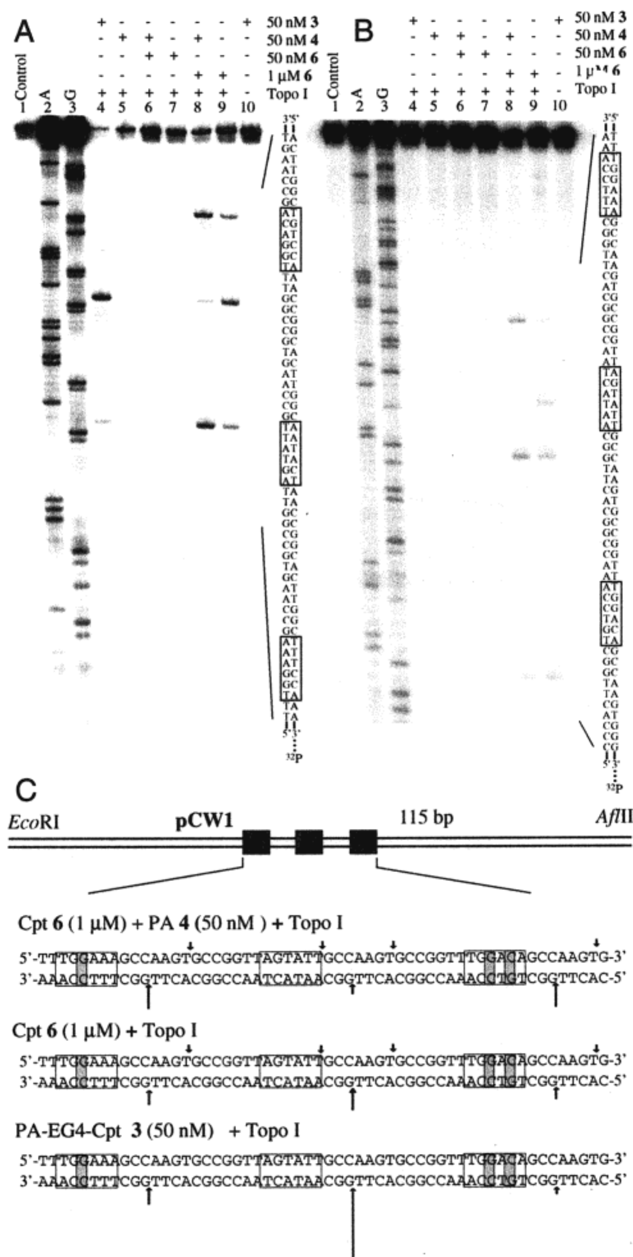

Figure 5. Topoisomerase I cleavage assay with polyamide−camptothecin conjugate **3** on the 115 base pair *Eco*RI/*Afl*II restriction fragments from plasmid pCW1: (A) 3′ ^{32}P end-labeled on the bottom strand. Lane 1, intact DNA; lane 2, A specific reaction; lane 3, G specific reaction; lane 4, 50 nM of conjugate **3** incubated with DNA followed by Topo I treatment; lane 5, 50 nM polyamide **4** with Topo I; lane 6, 50 nM camptothecin acid **6** and 50 nM polyamide **4** with Topo I; lane 7, 50 nM camptothecin acid **6** with Topo I; lane 8, 1 μM camptothecin acid **6** and 50 nM polyamide **4** with Topo I; lane 9, 1 μM camptothecin acid **6** with Topo I; lane 10, 50 nM conjugate **3** incubated with DNA. (B) 3′ ^{32}P end-labeled on the top strand. Lanes 1−10, same as panel A except on different DNA. (C) Illustration of the 115 bp restriction fragment with the position of the sequence indicated. Cleavage pattern for 1 μM camptothecin acid **6** and 50 nM polyamide **4** with Topo I; cleavage pattern for 1 μM camptothecin acid **6** with Topo I; cleavage pattern for 50 nM conjugate **3** (PA-EG4-CPT) with Topo I. Arrow length is proportional to cleavage yield. The arrow points to the cleavage between the bases. Boxes represent polyamide binding sites with gray boxes over mismatches.

(Figure 6). DNA cleavage yield decreased as the number of atoms in the linker region decreased: conjugate **3** (PA-EG4-Cpt) > conjugate **2** (PA-CA11-Cpt) > conjugate **1** (PA-CA5-

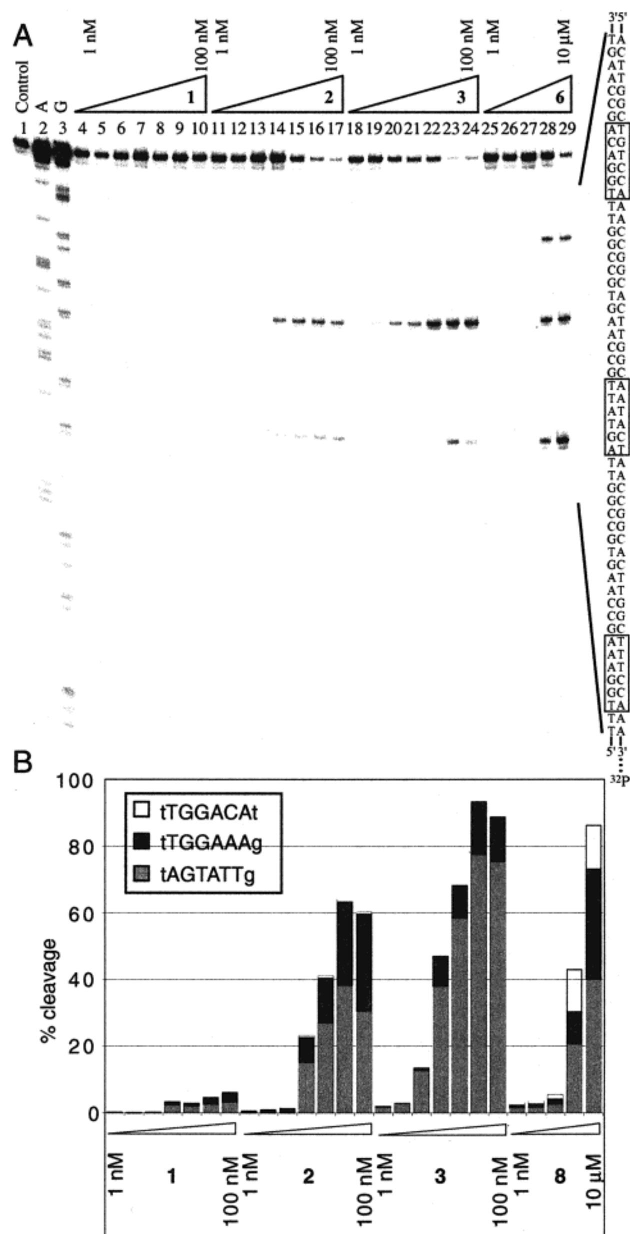

Figure 6. (A) Topoisomerase cleavage assay on conjugates **1**−**3** and camptothecin acid **6** on the *Eco*RI/*Afl*II restriction fragments from plasmid pCW1 labeled on the bottom strand: lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lanes 4−10, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM of conjugate **1**; lanes 11−17, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM of conjugate **2**; lanes 18−24, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM of conjugate **3**; lanes 25−29, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM camptothecin acid **6**. All ligands were incubated with DNA and treated with Topo I. (B) Bar graph of cleavage efficiency by conjugates **1**−**3**: the gray box represents the percent cleavage of 4 base pairs from polyamide binding site 5′-AGTATT-3′, the black box represents the percent cleavage of 4 base pairs from 5′-TGGAAA-3′, and the white box represents the percent cleavage of 4 base pairs from 5′-TGGACA-3′.

Cpt). The trend is consistent within the ligand concentration range of 1 to 100 nM. For each conjugate, cleavage yield increased with increasing ligand concentration until leveling off at 50 nM. The longer and flexible linker in conjugate **3** possibly allows the camptothecin moiety to adopt a favorable orientation in the binding pocket and could be the reason for its higher activity.

Implication for the Design of Topo I Hairpin Polyamide Conjugates. The results presented here reveal that polyamide−camptothecin conjugates can recruit Topo I to cleave single DNA sites in high yield. The difference in cleavage yields in conjugates **1**−**3** demonstrates the importance of linker length for conjugate activity. This sets the stage for the next step, which is to test whether conjugates targeted to the coding region of specific genes can regulate transcription elongation, which will be reported in due course.

Experimental Section

Materials. Fmoc-6-aminocaproic acid was purchased from Bachem, Fmoc-12-aminododecanoic acid was purchased from Aldrich, and Boc 17-amino-4,7,10,13-tetraoxa-heptadecanoic acid was a generous gift of Dr. Anna Mapp.[21] 10-Hydroxycamptothecin was purchased from JS International. DNA restriction fragment labeling protocols, DNase I footprinting, determination of equilibrium association constants, plasmid construction, and quantitation by storage phosphor autoradiography were as previously described.[14] Calf Thymus Topoisomerase I was obtained from Gibco BRL.

Preparation of Conjugates 1−3. ImPyPyPy-$(R)^{NH2}\gamma$-PyPyPyPy-β-Pam-Resin was synthesized in a stepwise fashion by solid-phase methods according to established protocols.[19] Fmoc-6-aminocaproic acid (25.5 mg, 75 μmol) and HBTU (28 mg, 75 μmol) were added in 2 mL of DMF and 1 mL DIEA and shaken for 1 h. The Fmoc group was deprotected with piperidine and the polyamide was cleaved off the resin with neat dimethylaminopropylamine in 16 h. A solution of camptothecin acid **6** (3.1 mg, 7.5 μmol), *N*-hydroxysuccinimide (0.85 mg, 7.5 μmol), and dicyclohexylcarbodiimide (16.7 mg, 81 μmol) in DMF (600 μL) was allowed to stir at room temperature for 2 h. ImPyPyPy-γ^{CA5NH2}-PyPyPyPy-β-Dp (3.3 mg, 2.5 μmol) and diisopropylethylamine (50 μL) in DMF (650 μL) were added and allowed to stir at room temperature for 9 h. The reaction was diluted with 0.1% trifluoroacetic acid in H_2O (6 mL) and purified by preparatory reverse-phase HPLC. Lyophilization of the pure fractions afforded conjugate **1**. For the synthesis of conjugate **2**, Fmoc-6-aminocaproic acid was replaced with Fmoc-12-aminododecanoic acid. For the synthesis of conjugate **3**, Fmoc-6-aminocaproic acid was replaced with Boc 17-amino-4,7,10,13-tetraoxaheptadecanoic acid. Yields and characterization: conjugate **1**, 0.4 mg, 10% recovery, MALDI-TOF [M + H] (monoisotopic), calcd 1753.90, obsd 1753.80; conjugate **2**, 0.5 mg, 13% recovery, MALDI-TOF [M + H] (monoisotopic), calcd 1838.06, obsd 1838.09; conjugate **3**, 1.6 mg, 24% recovery, MALDI-TOF [M + H] (monoisotopic), calcd 1916.08, obsd 1915.94.

Topoisomerase I Cleavage Experiment. Reaction mixtures (10 μL each) containing 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 15 μg/mL BSA, and 10 kcpm 3′-^{32}P-labeled 115 bp pCW1 *Eco*RI/*Afl*II restriction fragment were incubated with each polyamide−camptothecin conjugate **1**−**3** for 12 h at 22 °C. The Topoisomerase I reaction was initiated by the addition of 10 U of calf thymus Topo I 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 at 50 °C for 30 min. The reaction mixtures were ethanol precipitated and resuspended in 100 mM Tris-borate-EDTA/80% foramide loading buffer, denatured at 85 °C for 10 min, placed on ice, and immediately loaded onto an 85 °C denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1.5 h. The gels were dried under vacuum at 80 °C and then quantitated using storage phosphor technology.

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Supporting Information Available: Quantitative DNase I footprint titration experiments with polyamide−camptothecin conjugate **2** and **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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