FOR THE RECORD

DNA sequence selectivity of human topoisomerase I-mediated DNA cleavage induced by camptothecin

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Abstract: In probing the mechanism of inhibition of hypoxia inducible factor (HIF-1) by campothecins, we investigated the ability of human topoisomerase I to bind and cleave HIF-1 response element (HRE), which contains the known camptothecin-mediated topoisomerase I cleavage site 5′-TG. We observed that the selection of 5′-TG by human topoisomerase I and topotecan depends to a large extent on the specific flanking sequences, and that the presence of a G at the −2 position (where cleavage occurs between −1 and +1) prevents the HRE site from being a preferred site for such cleavage. Furthermore, the presence of −2 T/A can induce the cleavage at a less preferred TC or TA site. However, in the absence of a more preferred site, the HRE site is shown to be cleaved by human topoisomerase I in the presence of topotecan. Thus, it is implied that the −2 base has a significant influence on the selection of the camptothecin-mediated Topo I cleavage site, which can overcome the preference for +1G. While the cleavage site recognition has been known to be based on the concerted effect of several bases spanning the cleavage site, such a determining effect of an individual base has not been previously recognized. A possible base-specific interaction between DNA and topoisomerase I may be responsible for this sequence selectivity.

Keywords: human topoisomerase I; camptothecin; DNA cleavage site; HIF-1 response element

Introduction

Topoisomerase I (Topo1) is an important enzyme in clinical medicine, and is the sole target of camptothecin anticancer drugs.[1,2] Camptothecins reversibly stabilize the Topo1 cleavage complexes by inhibiting the religation of DNA during relaxation of supercoils. Structural data indicate that topotecan intercalates at the site of DNA cleavage, forming base stacking interactions with both the −1 (upstream) and +1 (downstream) base pairs, thereby displacing the reactive

Additional Supporting Information may be found in the online version of this article.

Abbreviations: hTopoI, human topoisomerase I; HIF-1, hypoxia inducible factor I; HRE, HIF-1 response element; VEGF, vascular endothelial growth factor.

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5′-OH of the cleaved strand away from the phosphotyrosine generated during cleavage.[3] The collision between the drug-stabilized cleavage complexes and advancing replication or transcription forks results in cytotoxic lesions that ultimately lead to cell apoptosis.

While Topo1 has long been known as the only target for camptothecins, camptothecin derivatives have recently been shown to be inhibitors of the hypoxia inducible factor-1 (HIF-1) pathway, based on a cell-based high-throughput screening aimed at identifying small molecule inhibitors of HIF-1.[4] It was subsequently demonstrated that Topo1 is required for the inhibition of HIF-1α protein activity by topotecan, and that Topo1 inhibitors with non-camptothecin chemical structures are also capable of inhibiting HIF-1α activity similarly to camptothecin analogues.[5] However, the exact mechanism of Topo1-mediated HIF-1 pathway inhibition by topotecan and other camptothecins has yet to be revealed. In an initial attempt to investigate this mechanism, we compared the DNA-binding properties of HIF-1 and Topo1 proteins. We noticed that the consensus DNA binding sequence of HIF-1 (HRE, 5′- RCGTG-3′) consists of the two base-step 5′-TG that is most commonly found in the camptothecin-stabilized Topo1 cleavage sites.[6–11] Thus we were interested in investigating whether the inhibition of HIF-1 activity by camptothecins is caused by the stabilization of topoisomerase I at the HIF-1 binding site, thereby preventing the DNA binding of HIF-1. Our data indicate that topotecan, a camptothecin derivative, can only induce a TopoI cleavage at the 5′-TG site within the HRE in the absence of other more preferred TopoI cleavage sites within the substrate. Previous studies have revealed that the region from −4 to +1 around TopoI cleavage sites display a consensus sequence preference, with a high preference for T at −1 and G at +1 and a small preference for A (or T) at position −4, G (or not T) at −3 and A or T at −2.[8–13] The presence of a +1 G has always been indicated to be more important than the −2 base for TopoI cleavage. Our study, on the other hand, provides the first direct experimental data that the base at the −2 position is sometimes more important than the +1 G in the determination of camptothecin induced cleavage by human TopoI. Based on the available structure data, the selectivity of the −2 base may in part be due to a possible hydrogen bonding interaction between its base pairing partner and the Lys532 of the enzyme.[3]

Results and Discussion

The preference for G at +1 of camptothecin-mediated cleavage site can be overcome by the flanking base at the −2 position

We have determined the cleavage specificities of human Topo1 using well-established cleavage assays that have previously been used for similar analyses.[9,12] These assays utilize oligonucleotides 3′-labeled by [α-^{32}P] cordycepin. Addition of a camptothecin analog and Topo1 to the DNA sample results in stabilization of the covalently bound Topo1 at a specific binding site with a nick in one strand of the DNA. The scissile DNA strands can be separated from the full-length oligonucleotide using denaturing polyacrylamide gel electrophoresis, and the length of the product is used to determine the cleavage site of Topo1.

In order to determine whether camptothecin-mediated Topo1 cleavage complex stabilization occurs within the HRE sequence, we first used an oligonucleotide sequence that was specifically designed to contain an HRE site (sequence W, 32-mer, 5′-AAGATCTAAAATACGTGGAAAAATTTTTAAAA-3′ with the HRE underlined). This sequence originated from an oligonucleotide derived from the Tetrahymena rDNA hexadecameric sequence,[14] which has previously been used to compare the inhibitory potency of camptothecin derivatives (Sequence X, 36-mer, 5′-GATCTAA AAGACTT(−1)↓G(+1)GAAAAATTTTTAAAAAAGCTC-3′, cleavage site indicated by an arrow).[15,16] The bases at the −2 and −5 positions of sequence X were replaced with G and T, respectively, to create the HRE site in sequence W [Fig. 1(A)].

The first cleavage assay was carried out with sequence W along with sequence X as a positive control. Topotecan was used to stabilize Topo1 cleavage complexes. While cleavage complex stabilization was observed with both oligonucleotides, it was interesting to note that the length of the cleaved DNA segment of sequence W was longer than expected [Fig. 1(B)]. Whereas cleavage of sequence W within the HRE sequence is expected to result in a 16-mer oligonucleotide, the observed length is ∼27 bases. This corresponds to cleavage of this sequence at a T$^{\downarrow}$C site, *i.e.*, 5′-AAGAT$^{\downarrow}$CTAAAATACGTGGAAAAATTTTTAAAA-3′, 11 bases upstream of the expected cleavage site. This result indicated that despite the presence of a TG site, the TopoI cleavage is prevented within HRE. The only differences between sequence W (HRE-containing) and X (known Topo I substrate) are the base replacements at the −2 (G *vs* T) and −5 (T *vs* G) positions and the addition of 5′-AA in W to create equal lengths of sequence on both sides of the TG cleavage site. Previous studies have revealed that only the region from −4 to +1 around TopoI cleavage sites display a consensus sequence preference.[8–13] In addition, T at the −5 position has been previously observed in cleavage assays with an SV40-derived oligonucleotide, which showed very high TopoI cleavage at the 5′-TG site.[9] It thus appears that the different −2 base between sequence W and X has a major influence on the observed difference in selectivity of the TG cleavage site by Topo1. Whereas previous reports indicate a sequence preference for A/T at the −2 position,[6,8,10,12] the +1 G has always been considered to be more important than the −2 base for TopoI cleavage. Our data show directly that a less-preferred −2 base (G in this

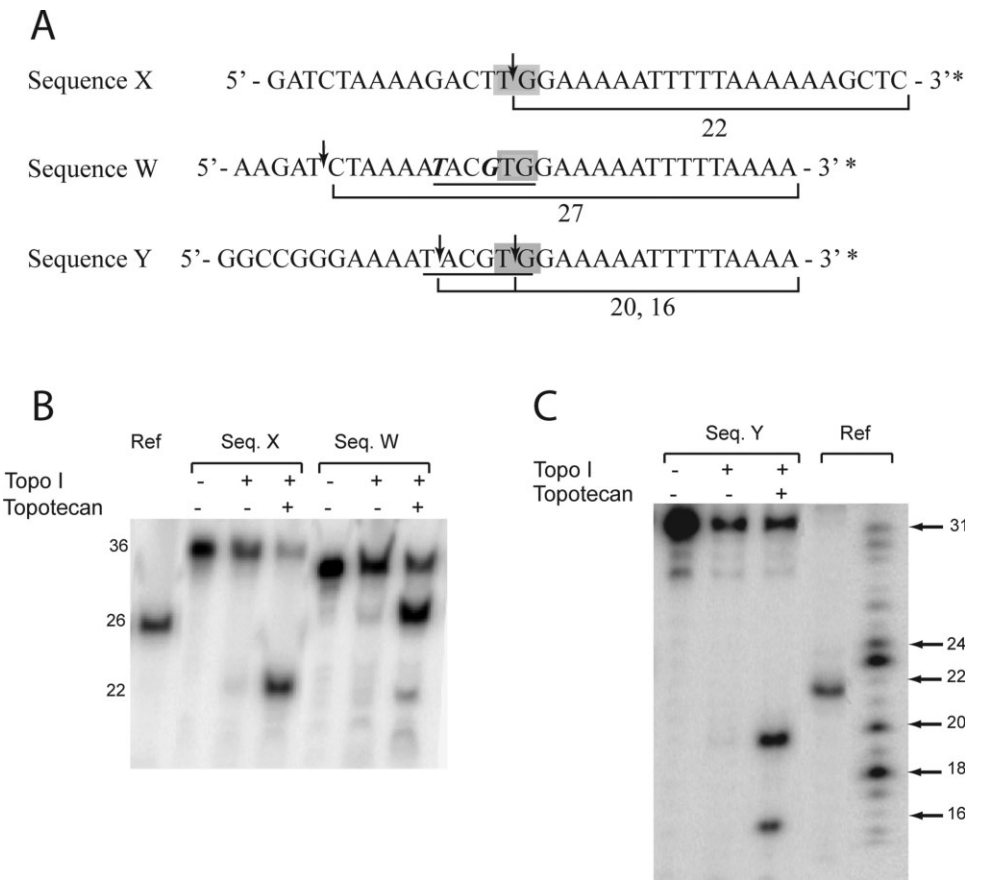

Figure 1. Cleavage Assays indicating the sites cleaved by TopoI in the presence of topotecan. (A) List of oligonucleotide sequences used in the cleavage assays. The potential cleavage sites containing a 5′-TG, the bases previously known to be essential for cleavage complex stabilization by camptothecin, are highlighted. The actual cleavage sites observed in our studies are indicated by downward arrows, and were in some cases different from the expected sites. The HRE sites are underlined. The brackets below each sequence demonstrate the fragments of DNA observed in the cleavage assays, and their lengths are as indicated. Sequence X: oligonucleotide derived from *Tetrahymena* rDNA and previously shown to have a preferred topoisomerase I cleavage site in the presence of camptothecins.[15,16] Sequence W: a truncated form of Sequence X with 2 bases (in bold Italic) replaced to form the HRE site. Sequence Y: a modified form of Sequence W containing the HRE, but lacking the observed TopoI cleavage site * denotes the labeled end (B) Assay with oligonucleotides containing either a canonical TopoI binding site (sequence X), or the HRE site (sequence W). (C) Assay with a modified oligonucleotide containing a HRE site (Sequence Y). For (B) and (C), numbers on the left/right side of the figure correspond to the length of oligonucleotides. Ref, an oligomer used for sequence length comparison.

case) can overcome the +1 G preference for Topo I cleavage and change the TopoI cleavage site from TG to TC. Such a negative influence of the −2 G base on the selection of the TopoI cleavage site has not been well established previously. Among all studies carried out to determine the sequence selectivity of TopoI in the presence of camptothecins, only a few identified a sequence preference for the −2 base.[6,8,10,12] A normalized probability calculation for +1 and −2 positions indicate that the presence of a +1 G is more important for cleavage by TopoI compared to the −2 base, while the preference of −2 A was only slightly higher than −2 G.[10] In addition, some studies have even shown a common occurrence of a −2 G in camptothecin-mediated cleavage sites.[7,12]

To determine whether Topo1 cleavage can occur within the HRE in the absence of a more preferred site in the flanking region, we repeated the above experiment with a different oligonucleotide lacking the preferred Topo1 cleavage site. We designed the sequence 5′-GGCCGGGAAAAT↓ACGT↓GGAAAAATTTT TAAAA-3′ (sequence Y, 32-mer), which differs from the sequence W in the first seven bases, and therefore lacks the Topo1 cleavage site observed with sequence W [Fig. 1(A)], but still contains the HRE site. We observed the anticipated cleavage between T and G of the HRE site in this sequence, giving rise to an oligonucleotide of 16 bases in length [Fig. 1(C)]. Interestingly, in addition to this expected cleavage, a stronger cleavage site adjacent to the HRE was observed

between T and A, giving rise to an oligonucleotide of 20 bases [Fig 1(C)]. Within this cleavage site, the −2 position is an A versus the G in the HRE site. Therefore it appears that the +1 A can be equally or more preferred by camptothecin-mediated Topo1 cleavage than the +1 G, in the presence of a less-preferred base at the −2 position. This observation reconfirms our findings that the Topo1 cleavage site preference is greatly influenced by the −2 position. To validate our assay conditions and to confirm the general applicability of previously reported data on base preferences for the −4 to +1 positions, we have also carried out a topotecan-mediated Topo1 cleavage study using a scrambled DNA sequence that contains multiple 5′-TG cleavage sites with different base combinations at the −4 to −2 positions (5′-TTTTCT↓GTT↓GGAGCCCACGT AT↓GCTTTTTTAA, with the potential 5′-TG cleavage sites indicated by arrows). Among the three possible cleavage sites, only the second one contains preferred bases in all of −4 to +1 positions, and was also the only cleavage site observed in our experiments (Supporting Information Fig. 1).

Of previous studies reporting the sequence specificity of camptothecin-mediated topoisomerase I cleavage, only those by Kjeldsen *et al.* and Pondarre *et al.* have analyzed cleavage sites of human topoisomerase I while others have used other sources of the enzyme.[7,17] However, it has been shown that the sensitivity to camptothecin differs significantly between evolutionarily distant species.[7] In comparisons of different topoisomerase enzyme types, slight differences in sequence specificity in the presence of camptothecins were observed between wheat germ and rat liver topoisomerase I,[8] as well as between wheat germ and calf thymus topoisomerase I.[10] Based on the limited data available on human topoI, a preference for T −1 and G +1 can be identified, but no conclusion can be made on the base preference at other flanking sites. Thus our data is likely to implicate new sequence selectivity information with regard to camptothecin-mediated cleavage complex stabilization by human topoisomerase I.

The role of Lys532 in the sequence selectivity of topoisomerase I

The crystal structures of Topo1 bound to DNA in the presence and absence of camptothecin show numerous interactions between the enzyme and DNA.[3,18,19] However, only one residue, the Lys532, was shown to make a base-specific contact with DNA, in spite of the sequence preferences of the enzyme which were experimentally observed in a number of studies.[3,18–21] Consequently, the molecular basis for the sequence preference from the −4 to +1 region has yet to be completely determined.

Since our studies above indicated that the presence of a G at the −2 position significantly affects the cleavage-site selectivity of Topo1, we were interested in identifying any potential contacts between the enzyme and the −2 base. In close examination of the topoisomerase I-DNA-topotecan ternary complex.[3] (PDB# 1K4T), it was noticed that the Nε of Lys532, located in the minor groove of DNA, is in fact at a possible hydrogen bonding distance with the N3 of adenine in the non-cleaved strand, which is complementary to the −2 T in the cleaved strand in this structure (see Fig. 2). In previous studies, the interactions of Lys532 with the O_2 of the −1 base of DNA, as well as with the camptothecins, were recognized.[3,18–21] Our analysis indicated that in addition to the O_2 of the −1 T, Nε of Lys532 may also form a hydrogen bond with the N_3 of the adenine opposite the −2 T, with a 3.36 Å distance between them. It should be noted that the minor groove faces of A:T and T:A base pairs are somewhat similar, but very different from those of G:C or C:G base pairs, in their steric shapes and H-bond donor-acceptor properties. This may explain the preference for A/T at −2, since even in the case of an A at −2 in the cleaved strand, the T in the opposite strand may still present a H bond acceptor in the minor groove

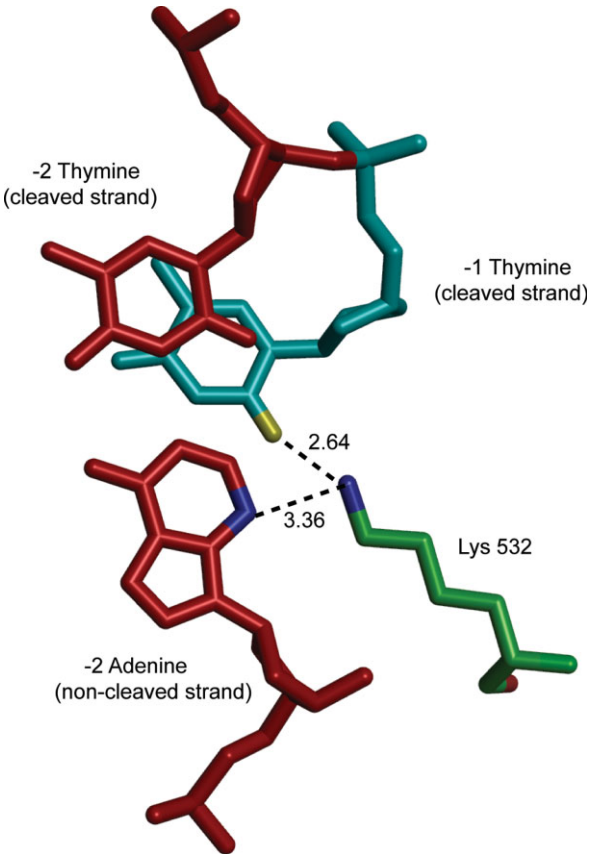

Figure 2. Hydrogen bond interactions between Lys532 of human topoisomerase I and −1 T or A complementary to −2 T (based on the PDB ID 1K4T.[3] The bases and amino acids are as labeled. The atoms involved in protein-DNA hydrogen bonding are highlighted with the nitrogens colored blue and the oxygen colored yellow. The respective hydrogen bond distances are as indicated.

for hydrogen bonding with Lys532. This is not the case with G:C/C:G base pairs at the −2 position. In addition, the different stacking interactions of G:C/C:G base pairs as compared to A:T/T:A may move the base away from interacting with Lys532, which can further disfavor a G at −2 position. Thus the potential hydrogen bond interaction we identified between the −2 A and Lys532 may be important in the sequence selectivity of topoisomerase I at the −2 position. This needs to be confirmed by further structural studies.

Materials and Methods

Drugs and chemicals

The water-soluble camptothecin analog topotecan was obtained from NCI and dissolved in water to obtain a final concentration of 0.5 m*M*. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Expression and purification of human Topo1

Human Topo1 was expressed in the baculovirus insect cell system as described previously.[22] Briefly, the protein was over-expressed in Sf9 cells and the protein extraction was carried out using pelleted nuclei. A solution containing Topo1 and other proteins was passed through consecutive phenyl sepharose, phosphocellulose, Mono-Q, Mono-S and POROS SP20 columns, and the peak Topo1 fractions were pooled. The resulting solution, which was detected to be >90% pure based on SDS-PAGE, was concentrated and dialyzed into storage buffer consisting of 50% glycerol, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, and 1 m*M* EDTA, and was stored at −20°C.

Topo1 cleavage assays

The oligonucleotides were custom-synthesized (Qiagen, Alameda, CA) and purified through a denaturing polyacrylamide gel. The upper strand was 3′ end-labeled using α-^{32}P-cordycepin (ddATP) (PerkinElmer, Wellesley, MA), and annealed with an excess of the unlabeled complementary strand by heating to 90°C for 5 min and then cooling slowly in annealing buffer (10 μ*M* Tris, pH 7.5, 10 m*M* $MgCl_2$, 1 m*M* DTT). The quantity of double-stranded DNA giving ~30,000 cpm radioactivity was incubated with 1 μg Topo1, with or without 50 μ*M* topotecan, in 10 μL reaction buffer (10 μ*M* Tris-HCl, 150 μ*M* KCl, 5 m*M* $MgCl_2$, 100 μ*M* EDTA, 15 μg/mL BSA). The final concentration of DNA substrate was ~0.15 μ*M* based on the UV absorbance of DNA sample prior to labeling, and the final concentration of TopoI was ~0.2 μ*M* as determined by Bio-Rad protein assay. After 30 min of incubation at room temperature, the reactions were stopped with 1 μL of 5% SDS, and heated for 5 min in 98% formamide buffer containing 10 m*M* NaOH. The samples were separated on a 16% denaturing polyacrylamide gel at 2000 V for 1 hr, and the dried gels were visualized by phosphorimaging.

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