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To cite this version:
Philippe Pourquier, Mary-Ann Bjornsti, Yves Pommier. Induction of Topoisomerase I Cleavage Complexes by the Vinyl Chloride Adduct 1, N 6 -Ethenoadenine. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 1998, 273 (42), pp.27245-27249. 10.1074/jbc.273.42.27245 . inserm-02438248

HAL Id: inserm-02438248
https://www.hal.inserm.fr/inserm-02438248
Submitted on 27 May 2021

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Induction of Topoisomerase I Cleavage Complexes by the Vinyl Chloride Adduct 1,N\textsuperscript{6}-Ethenoadenine*

(Received for publication, May 29, 1998, and in revised form, July 22, 1998)

Philippe Pourquier‡, Mary-Ann Bjornstål§, and Yves Pommier¶

From the *Laboratory of Molecular Pharmacology, Division of Basic Sciences, NCI, National Institutes of Health, Bethesda, Maryland 20892-4255 and the ¶Department of Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107-5541

We used purified mammalian topoisomerases I (top1) and oligonucleotides to study top1-mediated cleavage and religation in the presence of a potent carcinogenic adduct, 1,N\textsuperscript{6}-ethenoadenosine (eA) incorporated immediately downstream of a unique top1 cleavage site. We found that eA markedly enhanced top1 cleavage complexes when it was incorporated at the +1 position of the top1 cleavage. This enhancement was due to a reduction of the religation step of the top1 reaction. In addition, eA reduced the top1-mediated cleavage and decreased binding of the enzyme to DNA. We also studied the effects of the eA adduct on top1 trapping by camptothecin (CPT), a well known top1 inhibitor. CPT was inactive when eA was present at the +1 position. Alkylation of the top1 cleavage complex by 7-chloromethyl-10,11-methylenedioxy-7-CIME-MDO-CPT was also blocked by the eA adduct. Altogether, these results demonstrate that the eA carcinogenic adduct can efficiently trap human top1 and mimic CPT effects. Normal hydrogen bonding of the base pairs immediately downstream of a unique top1 cleavage site is probably essential for efficient DNA religation and binding of camptothecins in the top1 cleavage complex.

Vinyl chloride is a potent carcinogen. Its metabolites are reactive species that can induce cyclic adducts in DNA. Four adducts have been characterized: the 1,N\textsuperscript{2}-ethenodeoxynoguanosine (eG),\textsuperscript{1} the N\textsuperscript{2},3-ethenodeoxynoguanosine, the 3,N\textsuperscript{4}-ethenodeoxycytidin (eC), and the 1,N\textsuperscript{6}-ethenodeoxadenosine (eA).\textsuperscript{1} These adducts can also be generated by other exogenous sources such as vinyl carbamate, ethyl carbamate, and other P450-mediated oxidized two-carbon compounds (2,3). More recently, eA and eC have been detected in normal hepatocytes, suggesting their endogenous formation (4), probably through multiple reactive intermediates such as lipid peroxidation products (5). In vivo cytotoxicity and carcinogenic effects of etheno adducts have been linked to their mutagenic properties (6–9). For instance, eG causes G/A transitions and edA causes A/C transversions in vivo (7,9). When introduced into DNA, eA and eC are recognized and removed by human cell extracts at similar rates (10). Two separate DNA glycosylase activities have been reported for eA and eC excision (11), but the corresponding proteins have not been identified yet.

Topoisomerase I (top1) is important for DNA replication and transcription, because it can remove the torsional constraint along the DNA (12–14). Its role in DNA repair is less well established (15,16). Mammalian top1 cleaves one strand of the DNA and remains covalently attached to the 3’-end of the transient break. This catalytic intermediate is referred to as the top1 cleavage complex. This reaction is reversible, and in normal conditions, only a small fraction of the cleaved DNA is detectable. Top1 inhibitors (poisons) such as camptothecin (CPT) increase (trap) top1 cleavage complexes by inhibiting the religation step (17,18).

Both topoisomerase I and II can be trapped by DNA modifications such as mismatches, abasic sites, nicks, and gaps, when such lesions are in the vicinity of the enzyme cleavage site (19–22). In the present study, we used oligonucleotides containing an eA at the +1 position relative to the cleavage site and analyzed the effects on the top1-mediated DNA cleavage and religation and on top1 binding to DNA. We show for the first time that the carcinogenic adduct, eA, can trap top1 and mimic CPT effects. We also demonstrate that the eA adduct decreases top1 binding to DNA and the “on-rate” DNA cleavage. Finally, CPT activity was strongly inhibited when eA was in the immediate vicinity of a top1 cleavage site.

MATERIALS AND METHODS

Chemicals—High performance liquid chromatography-purified oligonucleotides were purchased from The Midland Certified Reagent Company (Midland, TX). α-32P-Cordycepin 5’-triphosphate was purchased from NEN Life Science Products and polyacrylamide from Bio-Rad. CPT and 7-chloromethyl-10,11-methylenedioxy-20(S)-camptothecin (7-CIME-MDO-CPT) were provided by Drs. Wani and Wall (Research Triangle Institute, Research Triangle Park, NC). Ten-millimolar aliquots of CPT or 7-CIME-MDO-CPT were stored at −20°C, thawed, and diluted to 1 mM in dimethyl sulfoxide (Me2SO) just before use.

Oligonucleotides Labeling and Annealing Procedures—3’ labeling was performed using terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) with α-32P-cordycepin as described previously (23). Labeling mixtures were subsequently centrifuged through a G-25 Sephacyr column to remove excess of unincorporated nucleotide. Radiolabeled single-stranded DNA oligonucleotides were annealed to the same concentration of unlabeled complementary strand in 1× annealing buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA). Annealing mixtures were heated to 85°C for 5 min and slowly chilled overnight to room temperature.

Top1 Purification—Overexpression of the human recombinant top1 I was performed in SF9 insect cells using a baculovirus virus construct containing the top1 full-length cDNA (24). Nuclear extracts were from infected SF9 cells 4 days after infection as described previously (20) and kept at −80°C before use. Human top1 was purified from nuclear extract using nickel-nitrilotriacetic acid-agarose beads (Qiagen, Santa Clarita, CA). 50 μl of beads were placed into microcentrifuge tubes and washed three times with 1 ml of wash buffer (50 mM Hepes, pH 7, 0.5 mM dithiorthiol, 10 mM MgCl2, 3 mM MnCl2, 50 mM KC1). 500 μl of nuclear extract were incubated with beads for 30 min at 4°C and

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‡ To whom correspondence should be addressed: Bldg. 37, Rm. 5D02, NIH, Bethesda, MD 20892-4255. Tel.: 301-496-5944; Fax: 301-402-0752; E-mail: pommier@nih.gov.

¶ The abbreviations used are: eG, 1,N\textsuperscript{2}-ethenodeoxynoguanosine; eC, 3,N\textsuperscript{4}-ethenodeoxycytidin; eA, 1,N\textsuperscript{6}-ethenodeoxyadenosine; top1, topoisomerases I; CPT, camptothecin; 7-CIME-MDO-CPT, 7-chloromethyl-10,11-methylenedioxy-7-CIME-MDO-CPT.

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Top1 Trapping by Ethenoadenine

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RESULTS

Top1 Is Reversibly Trapped by eA at the +1 Position of a Top1 Cleavage Site—In this study we have investigated the effect of eA on top1 activity in vitro. As shown in Fig. 1A, the eA bulky adduct differs from adenine by the presence of an additional ring resulting from a cyclization reaction of reactive species such as the vinyl chloride metabolite, chloroaacetalddehyde (26). We used purified oligonucleotides derived from the Tetrahymena ribosomal DNA sequence (27) and analyzed the effects of eA incorporation at the +1 position of the scissile strand (X in Fig. 1B) on top1-mediated DNA cleavage and religation using purified recombinant human enzyme. As shown in Fig. 1C, incorporation of eA led to approximately 6-fold increase of top1-mediated cleavage complexes (compare lanes 2). Top1 cleavage/religation equilibrium can be shifted toward religation by increasing the salt concentration in the reaction (28). In the case of the eA oligonucleotide, 0.5 M NaCl was able to reverse the top1-mediated cleavage complexes (Fig. 1C, left panel, lane 3).

Previous studies indicated that CPT interacted with the +1 base immediately 3’ to the top1 cleavage site (29) and increased the top1 cleavage complex (compare lanes 2 and 4 in the control panel) by inhibiting the relaxation step of the reaction (12, 17, 30). In the case of the eA oligonucleotide, CPT did not enhance top1-mediated DNA cleavage further (Fig. 1C, compare lanes 2 and 4 on the right panel), and the reaction was still reversible in the presence of 0.5 M NaCl (lane 5, right panel).

These results demonstrate that the carcinogenic adduct eA incorporated at the +1 position of a top1 cleavage site reversibly traps top1.

Kineti cs of Top1 Trapping by eA—We next compared the kinetics of top1 cleavage complex formation between the con-
control oligonucleotide (A:T) and the εA-containing oligonucleotide (εA:T) to investigate whether reversible trapping could be further enhanced by increasing the reaction time. As shown in Fig. 2A, cleavage complexes were formed within the first minute of incubation for both the normal and εA oligonucleotides, and no further increase was detected. Instead, a slight decrease of the cleavage complexes was detected with longer incubation, probably resulting from a decrease of the enzyme activity. In accordance with the results presented in Fig. 1C, the plateau value was 5–6-fold higher when εA was present at the +1 position (Fig. 2B). These results demonstrate that the reversible trapping of top1 by εA probably corresponds to an equilibrium that is reached quickly once top1 is added to the reaction.

εA at the +1 Position Inhibits the Formation of Top1-mediated DNA Cleavage—Because top1-mediated DNA cleavage/religation is an equilibrium process, reversible trapping of the enzyme by εA could possibly result from either an increase of the cleavage (on-rate) or a decrease of the religation (“off-rate”). In the following experiments, we separately investigated the effects of εA on each step of the top1 reaction. We first studied the DNA cleavage formation in “suicide” oligonucleotides containing either A or εA at the +1 position relative to the top1 cleavage site (see Fig. 3A). Under these conditions, top1-mediated cleavage was examined under single turnover conditions (25, 30). Once top1 cleaves the DNA, it remains bound to the 3′-end of the scissile strand in a covalent complex (suicide complex), and the 3′-labeled 6-mer distal product is released (Fig. 3B, arrowhead). In our reactions, the 5′-end of the non-scissile strand was phosphorylated prior to annealing to avoid formation of recombination products (20, 23). The kinetics experiment presented in Fig. 3B show a marked difference in the on-rate reaction between the two suicide substrates. Cleavage was slower in the oligonucleotide containing an εA at the +1 position than in the control (Fig. 3C). The apparent cleavage rate constant (kcl) was calculated. Cleavage was normalized to the end point value (lanes 8 in Fig. 3B) of 72 and 78% for the A:T (open squares) and εA:T (closed squares) oligonucleotide, respectively, and kcl was determined by fitting the data to the equation (100 − %Clnorm) = 100 e−kcl(t−t) (31). The kcl for the εA-containing substrate was approximately 3-fold less than the control (0.047 s−1 versus 0.15 s−1). These results indicate that the top1-mediated DNA cleavage rate (on-rate step) is reduced by the presence of εA at the +1 position of a top1 cleavage site.

εA at the +1 Position Inhibits Noncovalent Top1 Binding to DNA—To further determine whether presence of εA altered the top1-DNA interaction, DNA binding assays were performed. For this purpose, we used the catalytically inactive yeast top1 in which the active tyrosine in position 727 has been mutated to a phenylalanine (Y727F). This enzyme does not perform top1-mediated cleavage, but is still able to bind to the DNA (32, 33). We incubated 3′-labeled oligonucleotides containing either A (A:T) or εA (εA:T) at the +1 position in the presence of increasing amount of the yeast mutant top1 (Fig. 4). Y727F top1 was able to bind to both substrates, which resulted in the appearance of a slow migrating band corresponding to top1-DNA complexes in

![Image](65x392 to 281x729)

**Fig. 3.** εA:T at the +1 position of top1 suicide substrates decreases the cleavage on-rate. A, scissile strands were either A or εA at the +1 position were 3′-end-labeled and annealed to the normal 36-mer bearing a phosphate at the 5′-end. B, oligonucleotides were reacted at 25°C with purified top1 for different times (lanes 1–8, 5 s, 10 s, 30 s, 1 min, 3 min, 5 min, 10 min, and 30 min, respectively) before addition of 0.5% SDS (final concentration). C, gels were quantified using a PhosphorImager, and results were plotted as a function of time.

**Fig. 4.** εA at the +1 position decreases top1 binding. A, approximately 25 fmol of 3′-end-labeled oligonucleotides with either A (A:T) or εA (εA:T) at the +1 position (see Fig. 1B) were incubated with increasing amount of purified Y727F yeast mutant top1 for 5 min at 25°C. Lanes 1–4 correspond to 0, 0.75, 1.5, and 3.0 ng of the yeast mutant top1, respectively. Equal counts/min were loaded on a 6% native polyacrylamide gel in 0.25 × TBE, and top1-DNA complexes were separated from free DNA at 100 V for 2 h. B, top1-DNA complexes were quantified from the dried gel shown in A using a PhosphorImager. For each concentration of enzyme, top1 binding to εA:T was calculated relative to the A:T (control) oligonucleotide (binding of top1 to the control oligonucleotide was normalized to 1).
decreased by the presence of control DNA. These results demonstrate that top1 binding is decreased by approximately 4–5-fold compared with the oligonucleotide (Fig. 5A). As a comparison, we used the control oligonucleotide (Fig. 5B), which was incubated for 15 min at 25°C with purified top1 in the presence of increasing concentration of CPT before addition of 0.5% SDS. Lanes 1, DNA alone; lanes 2, +top1; lanes 3–6, +0.1, 1, 10, and 100 μM CPT, respectively. B, quantitations of the cleavage products in A using a PhosphorImager. Percentage of cleavage products was plotted as a function of CPT concentration. M, A:T (control) oligonucleotide; a, A:T oligonucleotide.

native polyacrylamide gels (Fig. 4A). Top1 binding to the eA-containing substrate was expressed relative to the binding to the control oligonucleotide (which was set to 1), and the ratio was determined for each concentration of enzyme (Fig. 4B). Top1 binding to the oligonucleotide with eA at the +1 position was reduced by approximately 4–5-fold compared with the control DNA. These results demonstrate that top1 binding is decreased by the presence of eA at the +1 position of the top1 site. They also suggest that reduction of the top1-mediated DNA cleavage on-rate (Fig. 3) is probably due to reduced binding of the enzyme to DNA.

eA Incorporation Mimics CPT Effects—Because the above data indicated that reduced top1 cleavage could not be responsible for top1 trapping by eA, we studied the influence of eA on the religation step (Fig. 5). For this purpose, we first incubated the normal and eA oligonucleotides (see Fig. 1B) with purified top1 for 15 min at 25°C to reach cleavage equilibrium (see Fig. 2). Religation was then studied after addition of 0.35 M NaCl (final concentration) for the indicated times before addition of 0.5% SDS. B, quantitation of the gel presented in A. Percentage of cleavage products were normalized relative to time 0. □, A:T oligonucleotide; ○, eA:T oligonucleotide; ■, inhibition of religation by CPT (10 μM) with the A:T oligonucleotide (not shown in A).

stratate that reversible trapping of top1 by eA is due to an inhibition of the religation step of the top1 reaction.

Lack of Effect of CPT in the Oligonucleotide with eA at the +1 Position—As shown in Fig. 1C, CPT did not increase further top1-mediated DNA cleavage when eA was present at the +1 position of the top1 site. We investigated this further by using different CPT concentrations (Fig. 6). Whereas CPT was active even at low concentration (0.1 μM) in the control oligonucleotide (A:T), it did not increase top1-mediated cleavage even at high concentration in the eA oligonucleotide (Fig. 6A, compare lanes 2 and 4 on the right panel). These results demonstrated that presence of eA at the +1 position relative to the top1 cleavage site prevents CPT activity.

eA at the +1 Position Prevents Alkylation of Guanine N3 by 7-CIme-MDO-CPT—Because CPT has been reported to interact directly with the +1 base from the top1 cleavage site (29), we tested whether the lack of effect of CPT on top1 cleavage

FIG. 5. eA incorporated at the +1 position decreases the kinetics of religation of top1-induced DNA cleavage complexes. 3’-end-labeled oligonucleotides with either an A (A:T) or eA at the +1 position (see Fig. 1B) were reacted with purified human top1. A, kinetics of religation were performed in the absence of CPT. DNA substrates (lanes a) were incubated with purified top1 for 15 min at 25°C. Reactions were then either stopped immediately with 0.5% SDS (lanes 0) or reversed at 4°C by adding 0.35 M NaCl (final concentration) for the indicated times before addition of 0.5% SDS. B, quantitation of the gel indicated times before addition of 0.5% SDS. Percentage of cleavage products were normalized relative to time 0. □, A:T oligonucleotide; ○, eA:T oligonucleotide; ■, inhibition of religation by CPT (10 μM) with the A:T oligonucleotide (not shown in A).

FIG. 6. eA at the +1 position prevents action of CPT. A, 3”-end-labeled oligonucleotides with either A (A:T) or eA (eA:T) at the +1 position (see Fig. 1B) were incubated for 15 min at 25°C with purified top1 in the presence of increasing concentration of CPT before addition of 0.5% SDS. Lanes 1, DNA alone; lanes 2, +top1; lanes 3–6, +0.1, 1, 10, and 100 μM CPT, respectively. B, quantitations of the cleavage products in A using a PhosphorImager. Percentage of cleavage products was plotted as a function of CPT concentration. M, A:T (control) oligonucleotide; a, eA:T oligonucleotide.

FIG. 7. eA at the +1 position prevents top1-mediated DNA alkylation by 7-chloromethyl-10,11-methylenedioxy- camptothecin. 3’-End-labeled oligonucleotides with A:T or eA:T at the +1 position (see Fig. 1B) were incubated with human purified top1 for 1 h at 25°C in the presence of increasing concentrations of 7-CIme-MDO-CPT. Lanes 1, DNA alone; lanes 2, +top1; lanes 3–6, +0.1, 1, 10, and 100 μM 7-CIme-MDO-CPT, respectively. The Arrow (X) indicates the alkylated DNA product (29).
was associated with alterations of the drug with the eA. For this purpose, we used the alkylating CPT, 7-ClMe-MDO-CPT. This drug alkylates the N3 position of the +1 guanine or adenine in the presence of top1 (29, 34). As reported previously, in the control oligonucleotide, 7-ClMe-MDO-CPT stimulated top1 cleavage complexes (Fig. 7, compare lane b with other lanes in the control panel) and formed the alkylated product that can be detected as a retarded band in denaturing polyacrylamide gel (Fig. 7, arrow). By contrast, when eA was incorporated at the +1 position, no increase of DNA cleavage was detectable with 7-ClMe-MDO-CPT. Moreover, the alkylation product was not detectable regardless of the concentration of 7-ClMe-MDO-CPT used. These results suggested that the lack of CPT activity when eA was present at the +1 position relative to a top1 cleavage site might be related to the altered structure of the +1 base pair.

**DISCUSSION**

In this study, we report for the first time that a potent carcinogenic adduct, eA can trap eukaryotic top1. eA incorporation results in altered base pairing with the thymine on the opposite DNA strand. Circular dichroism analysis demonstrates that the presence of the extra 4-membered ring suppresses the two hydrogen bonds (35). Our observation that eA can reversibly trap top1 is consistent with previous observations indicating that base mismatches at the +1 position can enhance top1 cleavage complexes (19, 36).

Reduced religation of top1-cleavable complexes appears to be the mechanism by which eA traps top1. Thus, carcinogenic adducts can mimic CPT effects. Once the DNA is cleaved by top1, perfect positioning of the adducts can mimic CPT effects. Once the DNA is cleaved by top1, perfect positioning of the adducts can mimic CPT effects. Once the DNA is cleaved by top1, perfect positioning of the adducts can mimic CPT effects. This positioning closely mirrors the expected DNA interaction with the thymines of the opposite strand. Circular dichroism analysis, which demonstrates the presence of the extra 4-membered ring, suppresses the two hydrogen bonds (35). Our observation that eA can reversibly trap top1 is consistent with previous observations indicating that base mismatches at the +1 position can enhance top1 cleavage complexes (19, 36).

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