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In vivo sequencing of camptothecin-induced topoisomerase I cleavage sites in human colon carcinoma cells

Corinne Pondarré[+], Dirk Strumberg, Akira Fujimori, Rosimar Torres-León and Yves Pommier*

Laboratory of Molecular Pharmacology, Division of Basic Sciences, Bldg 37, Rm 5C25, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA

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ABSTRACT

Camptothecin (CPT) is a specific topoisomerase I (top1) poison which traps top1 cleavable complexes; e.g. top1-linked DNA single-strand breaks with 5′-hydroxyl and 3′-top1 linked termini. CPT is also a potent anticancer agent and several of its derivatives have recently shown activity in the chemotherapy of solid tumors. Our aim was to apply the ligation-mediated polymerase chain reaction (LM-PCR) method to DNA extracted from CPT-treated cells in order to: (i) evaluate LM-PCR as a sensitive technique to detect *in vivo* CPT-induced cleavable complexes; (ii) investigate the frequency and distribution of CPT-induced DNA damage *in vivo*; and (iii) compare the distribution and intensity of cleavage sites *in vivo* and *in vitro*. This report describes a protocol allowing the sequencing of top1-mediated DNA strand breaks induced by CPT in the coding strand of the 18S rRNA gene of human colon carcinoma cells. CPT or its clinical derivatives, topotecan, CPT-11, SN-38, and 9-aminocamptothecin differed in their potency and exhibited differences in their DNA cleavage pattern, which is consistent with our previous *in vitro* studies [Tanizawa *et al.* (1995) *Biochemistry*, 43, 7200–7206]. CPT-induced DNA cleavages induced in the presence of purified top1 were induced at the same sites in the human 18S rDNA. However, the relative intensity of the cleavages were different *in vivo* and *in vitro*. Because mammalian cells contain ~300 copies of the rDNA gene per genome, rDNA could be used to monitor CPT-induced DNA cleavage in different cell lines and possibly in tumor samples.

INTRODUCTION

Topoisomerase 1 (top1) has been identified as the intracellular target of a new class of anticancer drugs, camptothecin (CPT) and its derivatives topotecan (Hycamtin^{R}), CPT-11 (Irinotecan^{R}) and 9-aminocamptothecin (1,2). The catalytic cycle of top 1 can be divided in four steps: (i) binding of the enzyme to DNA; (ii) cleavage of one strand with covalent bond to the 3′ phosphate terminus of the nicked intermediate leaving a 5′ OH group; (iii) complementary strand passage (or swivel) through the break; (iv) resealing and release of the enzyme [for recent reviews see (3,4)]. CPT and its derivatives inhibit the top1 catalytic cycle by preventing the resealing of the enzyme-mediated DNA single strand-breaks. Trapping of the top1 cleavable complexes represents the initial event of drug-induced cell death and plays a critical role in determining the cell sensitivity to top 1 inhibitors (5,6). This is illustrated by a recent study showing that in a panel of seven human carcinoma cell lines, drug cytotoxicity is better correlated with the frequency of CPT-induced cleavable complexes than top1 mRNA or protein levels (7). Thus, monitoring the amount of DNA breaks is important to determine the *in vivo* effects of camptothecins.

The alkaline elution and SDS–KCl techniques are commonly used to measure the frequency of cleavable complexes in cell culture systems (8–11). However, they both require radioactive labeling of the cellular DNA and therefore cannot be applied to quiescent cells or tumor samples. Also, these methods do not localize the drug-induced DNA damage. Prior to this report, CPT-induced DNA breaks had been sequenced *in vitro* in DNA oligonucleotides, SV40 and HIV-1 DNA and specific genes (12–18), and mapped *in vivo* but not at the nucleotide resolution (19–23).

A technique known as ligation-mediated polymerase chain reaction (LM-PCR) allows the amplification of specific DNA fragments of different lengths simultaneously. This is achieved by ligation of a double-stranded oligonucleotide linker to blunt-ended DNA fragments generated by DNA polymerase extension of a gene specific primer using a template DNA that has been chemically or enzymatically cleaved. Thus, all fragments have the same end and can be amplified by PCR using one pair of primers (24,25). The goal of the present study was to apply this

*To whom correspondence should be addressed. Tel: +1 301 496 5944; Fax: +1 301 402 0752; Email: pommier@nih.gov

+Present address: Hôpital Debrousse, 29 rue soeur Bouvier, 69322 Lyon cedex 05, France

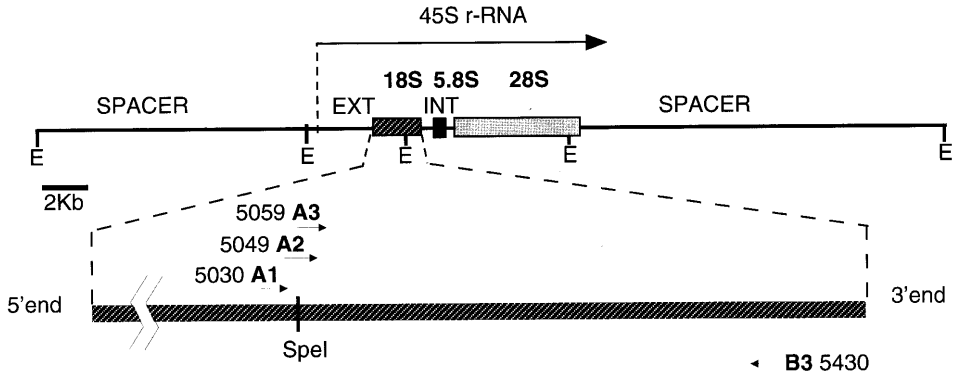

Figure 1. Map of a typical human rDNA gene repeat. A rDNA repeat unit (44 kb) is shown with the four segments defined by *Eco*R1 sites (E). Boxes give the position of sequences coding for the 18S, 5.8S and 28S rRNAs. The 18S rDNA is enlarged in the lower part of the figure and the primers used for LM-PCR (A1, A2 and A3) are shown. The A3–B3 PCR product was used as DNA substrate for *in vitro* cleavage reactions. Arrow at the top corresponds to the 45S transcriptional unit. Spacer, non transcribed region; Ext, external transcribed region; Int, internal transcribed region.

method to the sequencing of CPT-induced DNA breaks in drug-treated cancer cells.

We selected the transcribed regions of ribosomal DNA (rDNA) because: (i) there are ~300 copies of human rRNA genes per genome (26); and (ii) top1 has been reported to be enriched within the transcribed region of the human rRNA genes (23,27,28). Figure 1 shows a human rRNA gene repeat with its 31 kb spacer and 13 kb transcribed region which contains the 18S, 5.8S and 28S ribosomal subunit genes. Such repeats are arranged in tandem in the nucleolar organizer regions of the five acrocentric chromosomes (26).

MATERIALS AND METHODS

Cell lines

The SW 620 and HT29 cell lines were provided by Dr Dominic Scudiero (NCI, Frederick, MD) and grown in monolayer cultures using RPMI 1640 supplemented with 5% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Both cell lines were cultured at 37°C in 5% CO_2, trypsinized and passed 2–3 times per week.

Drugs, chemicals and enzymes

20-(*S*)-camptothecin lactone (camptothecin; CPT), 9-amino-20(*S*)-camptothecin (9-aminocamptothecin; 9-AC) and topotecan (TPT) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. CPT-11 (irinotecan^R)and SN-38 (7-ethyl-10- hydroxy-20(*S*)-camptothecin) were kindly provided by Dr Kiyoshi Terada (Yakult Honsha Co., Tokyo, Japan). Drugs were dissolved in dimethyl sulfoxide at a concentration of 10 mM. Further dilutions were made in water immediately before use. Vent$_R$ (exo–) DNA polymerase (2000 U/ml) and T4 polynucleotide kinase (10 U/µl) were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase (4 U/µl) was obtained from Stratagene whereas Klenow enzyme (2 U/µl) was from Boehringer Mannheim. Recombinant human top 1 was purified from baculovirus-infected sf9 insect cells as described (29,30).

Drug treatments and genomic DNA preparation

Exponentially growing cells were treated with 0.1 µM or 10 µM concentration of each drug. Following 1 h drug treatments in culture medium at 37°C, cells were washed twice with ice-cold Hank's Balanced Salt Solution (HBSS) containing the same amount of drug to avoid reversal of top1 cleavable complexes (31). To examine reversal of drug-induced DNA cleavage, treated cells were washed in drug-free ice-cold HBSS twice and allowed to grow in drug-free RPMI 1640 medium for 30 min. Following treatment, cells were lysed in 10 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, 0.6% SDS, pH 8.2. After overnight incubation at 37°C in the presence of proteinase K (3 mg/ml), DNA was isolated by phenol–chloroform extractions (three times) followed by dialysis against a solution containing 50 mM Tris–HCl and 10 mM EDTA at pH 8.

Ligation-mediated PCR

Oligonucleotide primers and linker. The oligonucleotide primers and linker were purchased from The Midland Certified Reagent Company (Midland, TX). PCR primers were chosen from the published sequence of human ribosomal DNA complete repeating unit (DDBJ/EMBL/GenBank accession number U13369) with numbers referring to GenBank genomic positions. Three nested primers for the 18S rDNA upper strand were used (Figs 1 and 2B): A1 for primer extension (corresponding to positions 5030–5050), A2 for PCR amplification (positions 5049–5074) and A3 for end-labeling extension (positions 5059–5080). The oligonucleotide linker consisted of a 25mer, L1 annealed to a 11mer L2 in 250 mM Tris–HCl, pH 7.7 (Fig 2B).

Formic acid sequencing of human genomic DNA. Untreated genomic DNA was sequenced according to the Maxam–Gilbert protocol. Thirty µl of formic acid (FA) were added to 30 µl of DNA in TE buffer for 4 min at room temperature. Reactions were terminated by ethanol precipitation. The chemically modified DNA was resuspended in 50 µl of freshly prepared 1 M piperidine, heated at 92°C for 30 min, ethanol precipitated in 0.3 M sodium acetate and lyophilized overnight to remove residual piperidine.

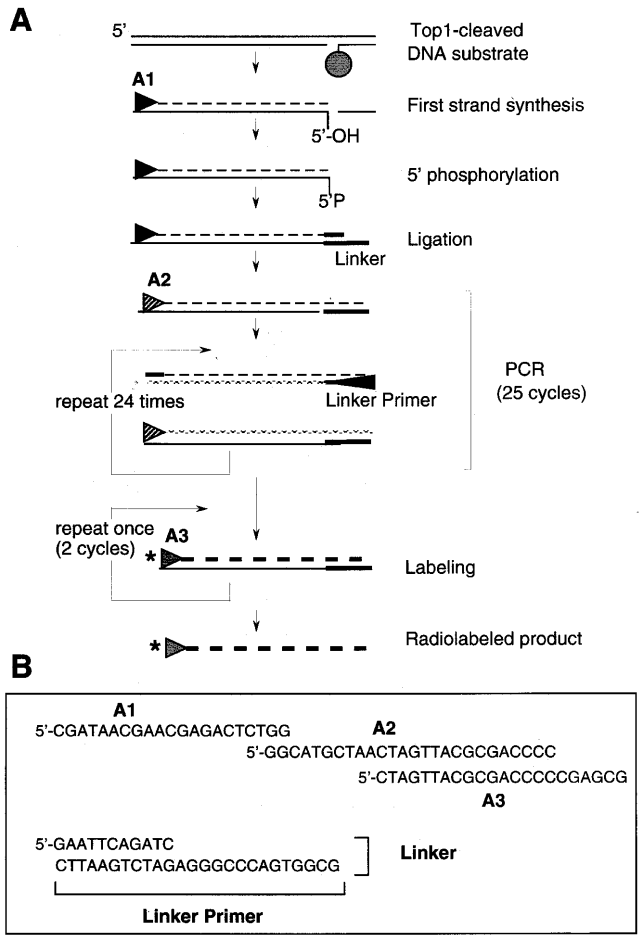

Figure 2. Schematic diagram of the ligation-mediated PCR (LM-PCR). (**A**) The 18s rRNA gene specific primer A1 is annealed to genomic DNA from CPT-treated or control cells. Extension is performed using Vent DNA polymerase to create a blunt end (first strand synthesis). After 5′-phosphorylation, the blunt end is ligated to a unidirectional blunt-ended linker (ligation). This product is the substrate for PCR using primer A2 (which anneals internally relative to primer A1) and a linker primer, which hybridizes to the ligated sequence (25 PCR cycles). Finally, a third 5′-end labeled internal primer, A3 is used for two additional extension cycles (labeling). The final product is complementary to the CPT-cleaved strand with the 25 base linker at the 3′-end. The star represents radioactive 5′ labeling of primer A3. The dotted circle at the top represents top1 covalently bonded to DNA. (**B**) Structure of the primers and linker.

Ligation-mediated PCR reaction steps. LM-PCR was performed in parallel with DNA from control and drug-treated cells, and with formic acid treated genomic DNA in five consecutive steps (Fig. 2A):

(i) First strand synthesis: genomic DNA (0.1 µg) was mixed in 25 µl of first strand mixture (40 mM NaCl, 20 mM Tris–HCl pH 8.9, 5 mM $MgSO_4$, 0.01% gelatin, 0.1% triton X-100, 0.3 pmol of primer A1, 250 µM of all four deoxyribonucleoside triphosphates). Vent DNA polymerase (0.5 U) was then added. Denaturation at 98°C for 5 min was followed by primer A1 annealing at 60°C for 30 min. The first primer A1 extension to the variable CPT-induced DNA cleavage sites was carried out at 76°C for 10 min. This generated a series of blunt ended duplex DNAs with 5′-OH at the top 1 cleavage sites.

(ii) Subsequent phosphorylation of the 5′-OH termini using T4 polynucleotide kinase added the 5′ phosphate required for ligation.

(iii) Ligation: after precipitation, the DNA was resuspended in 30 µl of ligase buffer (13 mM NaCl, 75 mM Tris–HCl, pH 7.5, 1.5 mM $MgSO_4$, 0.003% gelatin, 12 mM $MgCl_2$; 33 mM DTT; 83 µg/ml BSA) and 25 µl of reaction mixture (10 mM $MgCl_2$, 20 mM DTT, 3 mM ATP, 50 µg/ml BSA, 4 µM linker in 50 mM Tris–HCl, pH 7.7 and 4 U T4 DNA ligase). Samples were ligated overnight at 17°C and ethanol precipitated in 0.1 M sodium acetate in the presence of 10 µg yeast tRNA.

(iv) PCR: the fragments were amplified in 50 µl of 40 mM NaCl, 20 mM Tris–HCl pH 8.9, 5 mM $MgSO_4$, 0.01% gelatin, 0.1% triton X-100, 200 mM dNTPs, 5 pmol of primer A2, 5 pmol of linker primer (25mer) with 1 U Vent DNA polymerase. Twenty-five PCR cycles were as follows: 1 min at 95°C, 2 min at 67°C and 3 min at 76°C with a 5 s extension of the last step for each cycle. For the first cycle, samples were denaturated at 95°C for 4 min. Extension time for the last cycle was 10 min.

(v) Labeling: amplified DNA products were labeled with 10 µl of radioactive labeling mix (40 mM NaCl, 20 mM Tris–HCl, pH 8.9, 5 mM $MgSO_4$, 0.01% gelatin, 0.1% triton X-100, 200 µM dNTPs, 2.3 pmol 5′-^{32}P-end-labeled primer A3 and 1 U Vent DNA polymerase). Two cycles were performed as follows: 95°C for 1 min, 68°C for 2 min, 76°C for 3 min with a 10 min extension time in the last cycle. After filtration through a sephadex G50 column, the samples were ethanol precipitated and resuspended in loading dye. The position of the *in vivo* cleavage sites was determined by running DNA sequencing reactions of the A3–B3 PCR product on the same gel. A correction (25 nucleotide difference) was made to account for the linker primer present at the end of the *in vivo* generated fragments.

CPT-induced DNA cleavage in 18S rDNA in the presence of purified top1

Primer A3 (Fig. 2B) and B3 (5′-GCCGTGGGCCGACCCCGG-CG, positions 5430–5410) were used to amplify a 372 bp A3–B3 fragment between position 5059 and 5430 (Fig. 1). All amplification reactions consisted of 0.1 µg of total control genomic DNA, appropriate primers (5 pmol each), the four deoxynucleotides (250 µM each), 1 U of Vent-DNA polymerase and its supplied buffer (New England Biolabs, Beverly, MA). Thirty PCR cycles were performed. Primers A3 and B3 were annealed at 68°C. The 5′-end labeling of the A3–B3 fragment was obtained by PCR using 5′-end-labeled primer B3. Substrate DNA was then incubated with or without CPT in 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 µg/ml bovine serum albumin in the presence of recombinant human top1 at 37°C for 30 min. Reactions were stopped by adding SDS to a final concentration of 1% and digested with proteinase K (0.5 mg/ml for 1 h at 50°C).

DNA electrophoresis

DNA fragments were separated by electrophoresis in 7% polyacrylamide wedged gels. The gels were then autoradiographed or analysed using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Cleavage sites were positioned by comparison with a purine ladder.

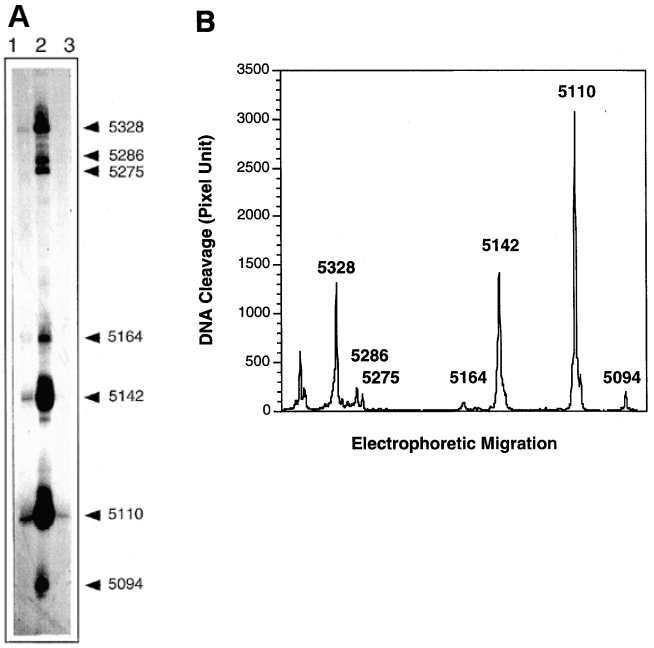

Figure 3. *In vivo* sequencing of CPT-induced DNA cleavage sites in the 18S rDNA of human colon carcinoma SW620 cells: autoradiography of LM-PCR products. (**A**) Control DNA from untreated cells was without any signal. Lanes 1 and 2: DNA from cells treated with 0.1 and 10 μM CPT, respectively. Lane 3: reversal of CPT-induced DNA breaks. CPT (10 μM for 1 h) was removed from cell culture 30 min before DNA extraction. Numbers to the right correspond to genomic positions of the DNA breaks on the lower strand (DDBJ/EMBL/GenBank U13369). Top1 is linked to the nucleotide corresponding to the indicated number. The positions of the cleavage sites at the top of the gel (5275–5328) are ± 1 nucleotide. (**B**) Intensity profile of the cleavage sites using the Phosphorimager ImageQuant software (Sunyvale, CA).

RESULTS

Detection of *in vivo* CPT-induced cleavable complexes using LM-PCR

Figure 2 describes the LM-PCR method and primers used to sequence the camptothecin-induced cleavage sites in the coding strand (transcribed; lower strand in GenBank) of the human 18S rDNA. A previous report indicated that top1 is preferentially associated with the coding strand of transcribed genes (22). LM-PCR was successfully applied to DNA extracted from both control and CPT-treated cells. Details are given in Materials and Methods for the LM-PCR protocol developed for the present experiments. Figure 3 illustrates the results of a typical experiment using 0.1 μg of genomic template DNA (equivalent to ~10^4 cells). No cleavage was detected in DNA from control cells indicating that amplification was specific for top1-induced DNA breaks. Treatment with 0.1 μM CPT induced several cleavage sites that were intensified at 10 μM CPT, indicating a dose-dependent effect. Also, 10 μM CPT induced additional sites that were not as intense as those that were already detectable at 0.1 μM. Overall, seven cleavage sites were identified within an 18S rRNA region encompassing ~400 nucleotides (from position 5059) with one site of high intensity (position 5110) (Fig. 3B). The same pattern of distribution and intensity of CPT-induced cleavage sites was observed in another colon carcinoma cell line, HT-29 (data not shown).

We examined the reversibility of CPT-induced cleavable complexes. Cells were treated with 10 μM CPT, and DNA breaks were examined 30 min after drug removal. As expected from previous data in genomic DNA (28,31), almost complete reversal was observed. The LM-PCR method was sensitive enough to detect persistent cleavage at some sites (Fig. 3). These results were reproducible in three independent experiments.

Comparison of CPT derivatives

We next compared CPT derivatives presently used in the clinic: topotecan (Hycamtin^R), 9-aminocamptothecin, CPT-11 (Irinotecan^R), and its active metabolite, SN-38. Figure 4 shows representative sequencing gel autoradiographies. Cleavages were generally induced by all drugs at similar sites with the exception of CPT-11. CPT-11 was inactive at 0.1 μM, and induced more diffuse and less intense cleavage at 10 μM (lane 9) compared with the other camptothecins. The cleavage patterns of the other CPT derivatives were comparable with some intensity differences. For instance, one cleavage site indicated with an asterisk (Fig. 4) was consistently observed with 9-aminocamptothecin and was weaker for CPT and the other derivatives. Finally, SN-38 was the most active compound at low drug concentration, consistent with previously published *in vitro* data (32).

In vitro CPT-induced DNA cleavages in human 18S rDNA

In order to study the location of top 1-mediated cleavage sites induced by CPT (10 μM) *in vitro*, the 5′-end labeled A3–B3 DNA fragment (Fig. 1)was amplified and this enabled us to identify top1-mediated breaks in the genomic region between nucleotides 5059 and 5430 (Fig. 5).

Strong cleavage sites *in vivo* (positions 5110 and 5142) were also found *in vitro* with relative high intensity. In addition, two moderate cleavage sites *in vivo* (positions 5286 and 5275) and one cleavage site at position 5164 were also found *in vitro* with similar intensity. The strong *in vivo* signal at position 5328 was weak *in vitro*. However, the strong 5331 site detected *in vitro* might be part of the *in vivo* band 5328 because this *in vivo* signal was in the upper part of the gel which was less well resolved.

Beside these common cleavage sites *in vivo* and *in vitro*, the *in vitro* assay presented a number of additional cleavages. Most of them were even detectable after the top 1-reaction alone without any inhibitor. A number of additional *in vitro* signals did only appear after top1-trapping with CPT (i.e. position 5331, 5358, 5365, 5374). Thus, the position of the *in vivo* and *in vitro* sites was generally consistent but the relative intensities were different. A number of additional *in vitro* signals were not present *in vivo*, suggesting that chromatin structure influence top 1 activities.

DISCUSSION

To our knowledge, this is the first report of sequencing the DNA cleavage sites induced by camptothecins in genomic DNA. The presented cleavage sites correspond to top1-mediated DNA breaks since similar sites were induced *in vitro* and *in vivo*, and the cleavage was readily reversible upon CPT removal. Several cleavage sites (5094, 5110, 5275 and 5286) match the CPT preferential sequence 5′-T^G, e.g. with a T at the 3′-DNA terminus of the top1-induced DNA break, to which the enzyme

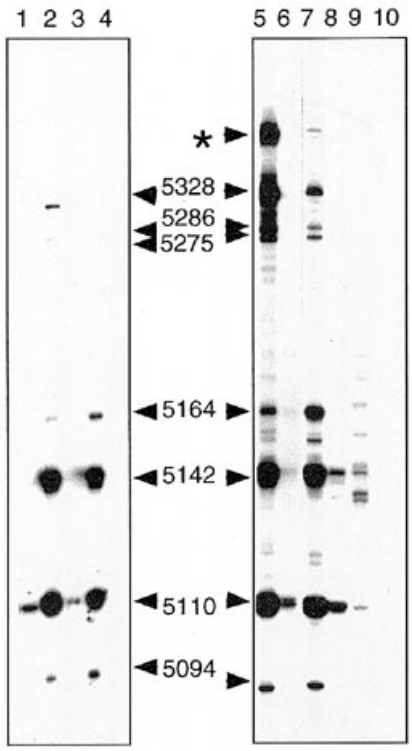

Figure 4. Comparison of the *in vivo* cleavage sites induced by CPT and its clinically used derivatives in the 18S rDNA using LM-PCR. Genomic DNA was extracted from SW620 cells after 1 h treatments with 0.1 μM (lanes 1, 3, 6, 8 and 10) or 10 μM drug concentrations (lanes 2, 4, 5, 7 and 9). The lanes of the autoradiography pictures are: lanes 1 and 2, CPT; lanes 3 and 4, topotecan; lanes 5 and 6, 9-aminocamptothecin; lanes 7 and 8, SN-38; and lanes 9 and 10, CPT-11. Numbers correspond to genomic positions of the DNA breaks. The positions of the cleavage sites at the top of the gel were consistently more intense in the case of 9-aminocamptothecin.

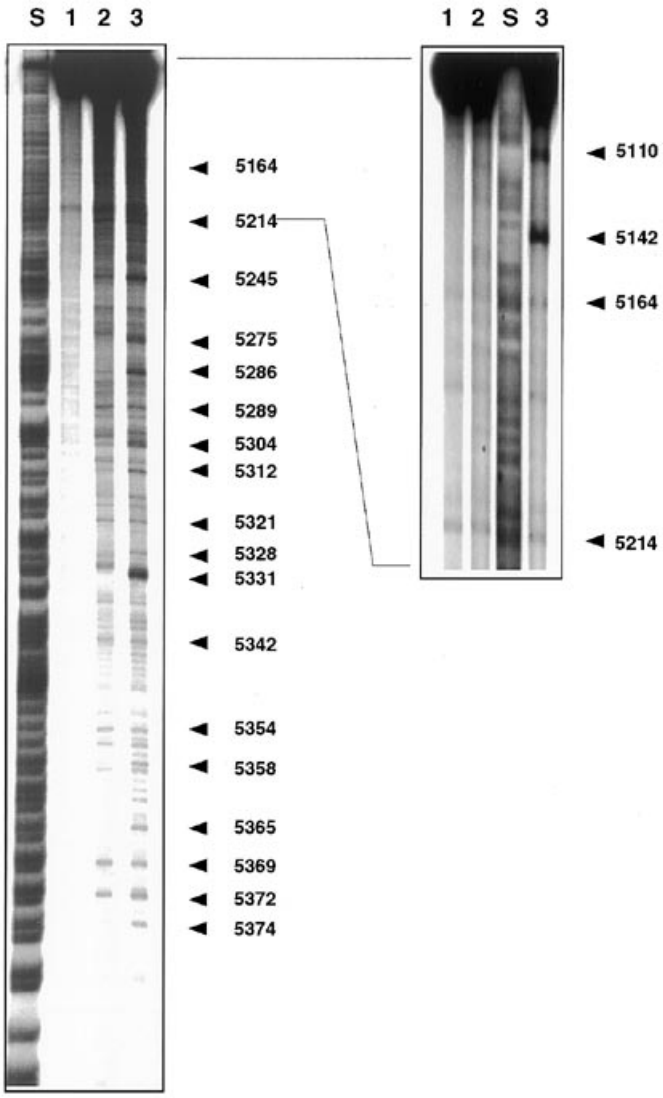

Figure 5. *In vitro* sequencing of CPT-induced top1 cleavage sites in the lower strand of 18S rDNA (DDBJ/EMBL/GenBank U13369). The purified DNA fragments were incubated with 10 μM CPT for 30 min at 37°C in the presence of recombinant human top 1. Reactions were stopped with SDS and proteinase K (1% and 0.5 mg/ml, respectively). The 5′-end-labeled A3–B3 PCR fragment was used (see Fig. 1, bottom). Lane 1, DNA alone; lane 2, + top1; lane 3, + top1+ CPT; and lane S, purine ladder.

is covalently linked and a G at the 5′-OH DNA terminus, which is specific for CPT (14,16). Two other sites (5142 and 5164) had a T at the 3′-DNA terminus of the top I-induced DNA break.

Previous studies in genomic DNA were performed using the end-labeling technique and did not provide nucleotide level resolution (19–23). Our *in vivo* sequencing is in agreement with the report of Culotta *et al.* (23) showing the high frequency of camptothecin-induced sites in transcribed regions of the rDNA genes. We find that within the 400 bp region examined the two strongest *in vivo* cleavage sites were ~200 bp apart (sites 5110 and 5328).

Because mammalian cells contain ~300 copies of the rDNA gene per genome, rDNA could be used to monitor CPT-induced DNA cleavage in different cell lines and possibly in tumor samples to monitor the cellular response to camptothecins.

Further studies are warranted to determine whether CPT-induced DNA cleavage sites can be mapped in single copy genes. The published *in vitro* cleavage sites sequenced in the human c-*myc* proto-oncogene (33,34) and top1 gene (16) may serve to guide the design of primers for LM-PCR based *in vivo* sequencing. A limitation of LM-PCR is that the method presently described cannot be used to sequence top2-induced DNA breaks because the 5′-DNA termini of such breaks are covalently linked to top2 and cannot be directly ligated.

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