# Substitution of 5-methylcytosines for cytosines enhances the stability of topoisomerase I-DNA complexes and modulates the sequence selectivity of camptothecin-induced DNA cleavage

Carolina Carrasco[1,a], Michael J. Waring[b], Christian Bailly[a,*]

[a] *Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret et INSERM U-124, IRCL, Place de Verdun, 59045 Lille Cedex, France*
[b] *Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK*

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**Abstract We have investigated the binding and cleavage of DNA by human topoisomerase I using a 160 bp restriction fragment containing either natural bases or 5-methylcytosine residues in place of cytosines. Experiments were performed in the presence and absence of the antitumour drug camptothecin which specifically inhibits topoisomerase I. Replacement of all cytosines with 5-methylcytosine residues (i) reinforces the enzyme-DNA interaction, (ii) enhances the stability of topoisomerase I-DNA complexes and (iii) modulates the sequence selectivity of camptothecin-induced DNA cleavage. The methyl group exposed in the major groove of the double helix is identified as a critical element for the interaction between topoisomerase I and DNA.**


*Key words:* Topoisomerase; Methylcytosine; Camptothecin

## 1. Introduction

Topoisomerase I is an essential DNA-cleaving enzyme involved in DNA replication, transcription, genetic recombination and other fundamental processes [1]. In addition, this ubiquitous enzyme is a privileged target for antitumour and antiviral drugs [2]. The reaction between double-stranded DNA and topoisomerase I produces a covalent 3′-phosphorotyrosyl adduct, usually referred to as the cleavable complex. Under physiological conditions, the DNA cleavage and ligation reactions catalysed by the enzyme are tightly coordinated and the covalent intermediate is barely detectable. The cleavage is coupled with the religation to restore continuity to the DNA duplex. A number of drugs such as the antitumour alkaloid camptothecin can convert topoisomerase I into a cell poison by blocking the religation step, thereby enhancing the formation of persistent DNA breaks responsible for cell death [3].

The topoisomerase I-mediated cleavage of DNA is sequence-selective and occurs predominantly on the 3′ side of thymine residues. Camptothecin preferentially traps a subset of the enzyme cutting sites, namely those containing a guanine immediately 3′ to the cleaved bond [4,5]. The drug binds at the interface of the enzyme-DNA complex and interacts with the $G^{+1}$ residue [6]. The methyl group of thymine which protrudes into the major groove is therefore expected to play an important role in the sequence-selective DNA recognition process. Studies with synthetic oligonucleotides have shown that both the removal of the $CH_3$ group from T (by substituting uridine for thymidine) or its addition onto C (replacement of cytosine with 5-methylcytosine) can affect the cleavage activity of the enzyme. The cutting reaction can be stimulated or strongly inhibited depending on the position of the U or 5-Me-C residues with respect to the cleavage site [7,8]. These studies, and others performed with type I topoisomerase of vaccinia virus [9], strongly suggest that interactions within the major groove of DNA may be critical for the cleavage activity of the enzyme.

To investigate further the mechanism of interaction between topoisomerase I and the major groove of DNA, we have compared the binding of the human enzyme to DNA containing either natural bases or 5-methylcytosine residues in place of cytosines. The 160 bp DNA fragment used as a substrate was elaborated by PCR using dATP, dTTP, dGTP and either dCTP or 5-methyl-dCTP, chosen at will. The experiments described in this paper show that the replacement of all cytosines with 5-methylcytosine residues (i) reinforces the enzyme-DNA interaction, (ii) enhances considerably the stability of topoisomerase I-DNA complexes and (iii) modulates the sequence selectivity of camptothecin-induced DNA cleavage.

## 2. Materials and methods

### 2.1. Chemicals and enzymes

Camptothecin (Sigma Chemical Co., La Verpillière, France) was dissolved in dimethyl sulphoxide at 1 mM and then further diluted with water. [$^{32}$P]γ-ATP was obtained from Amersham and 5-methyl-dCTP was from Pharmacia. Restriction endonucleases *Eco*RI and *Ava*I (Boehringer), *Taq* polymerase (Promega) and T4 polynucleotide kinase (Pharmacia) were used according to the suppliers' recommended protocol in the activity buffer provided.

### 2.2. Polymerase chain reaction and labelling of DNA

The 160 base pair *tyrT*(A93) fragment used as a template was isolated from the plasmid pKMp27 [10] by digestion with restriction enzymes *Eco*RI and *Ava*I. The protocol used to incorporate 5-methylcytosine residues into DNA was identical to that previously used to incorporate 7-deazapurine or 2,6-diaminopurine residues [11,12]. Briefly, PCR reaction mixtures contained 10 ng of *tyrT*(A93) DNA template, 1 μM each of the appropriate pair of primers (one with a 5′-OH and one with a 5′-$NH_2$ terminal group) required to allow 5′-phosphorylation of the desired strand, 250 μM of each appropriate dNTP or 5-methyl-dCTP instead of dCTP, and 5 units of *Taq* polymerase in a volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, and 1.5 mM $MgCl_2$. To prevent unwanted primer-template annealing before the cycles began, the reactions were heated to 60°C before adding the *Taq* polymerase. After an initial denaturing step of 3 min at 94°C, 20 amplification cycles were

*Corresponding author. Fax (33) 320 16 92 29.
E-mail: bailly@lille.inserm.fr

[1] Present address: Departamento de Bioquimica y Biologia Molecular, Universidad de Alcalà de Henares, Madrid, Spain.

*Abbreviations:* SDS, sodium dodecyl sulphate


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performed, each cycle consisting of the following segments: 94°C for 1 min, 37°C for 2 min, and 72°C for 10 min. After the last cycle, the extension segment was continued for an additional 10 min at 72°C, followed by a 5 min segment at 55°C and a 5 min segment at 37°C. Samples were then purified on a 6% non-denaturing polyacrylamide gel. The purified PCR products were 5′-end labelled with [γ-$^{32}$P]ATP in the presence of T4 polynucleotide kinase and then the labelled DNA was again purified by 6% polyacrylamide gel electrophoresis. Finally, the labelled DNA was resuspended in 10 mM Tris-HCl, pH 7.0 buffer containing 10 mM NaCl.

*2.3. Topoisomerase I binding to DNA*

$^{32}$P-Labelled *tyrT* DNA was incubated with 5 units of human topoisomerase I in the absence or presence of camptothecin for 45 min at 37°C. Binding reactions were performed in the topoisomerase I buffer containing 20 mM Tris-HCl pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, in a 20 μl reaction volume. Samples were analysed on a 6% non-denaturing polyacrylamide gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM $Na_2$-EDTA, pH 8.3).

*2.4. Sequencing of topoisomerase I-mediated DNA cleavage sites*

Each reaction mixture contained 2 μl of $^{32}$P 3′-end labelled DNA (~1 μM), 5 μl of water, 2 μl of 10×topoisomerase I buffer, and 10 μl of 20 μM camptothecin. After 10 min equilibration, the reaction was initiated by addition of 2 μl human topoisomerase I from TopoGen Inc. (Columbus, OH). Samples were incubated for 45 min at 37°C prior to adding SDS to 0.25% and proteinase K to 250 μg/ml in order to dissociate the drug-DNA-topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 μl of formamide-TBE loading buffer, denatured at 90°C for 4 min and chilled in ice for 4 min prior to loading on to the sequencing gel. DNA cleavage products were resolved by electrophoresis under denaturing conditions in polyacrylamide gels (0.3 mm thick, 8% acrylamide containing 8 M urea). Electrophoresis was performed for about 2 h at 60 W in TBE buffer. Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C, and analysed using a phosphorimager (Molecular Dynamics 445SI, ImageQuant version 4.1 software). Each resolved band on the autoradiograph was assigned to a particular bond within the DNA fragment by comparison of its position relative to guanine markers generated by treatment of the DNA with dimethylsulphate followed by piperidine-induced cleavage (G track).

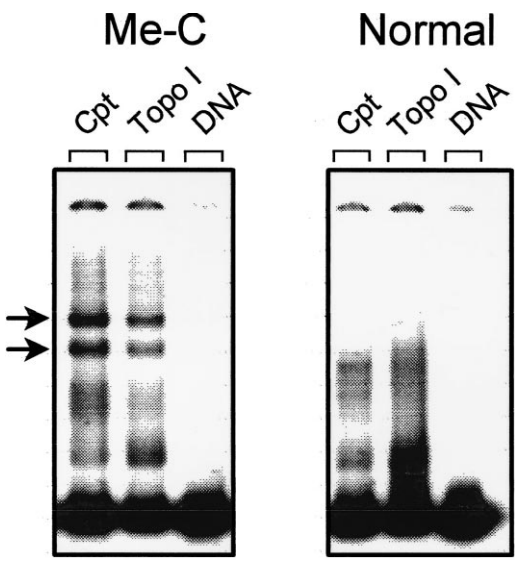


Fig. 1. Binding of topoisomerase I to DNA containing cytosine or 5-methylcytosine residues. Both normal and Me-C DNA were incubated for 45 min at 37°C with 4 units of human topoisomerase I in the absence (lanes Topo I) or presence (lanes Cpt) of camptothecin at 10 μM. 5 μl of a solution containing 0.1% electrophoresis dyes and 40% glycerol were added prior to loading the samples on a 6% non-denaturing polyacrylamide gel.

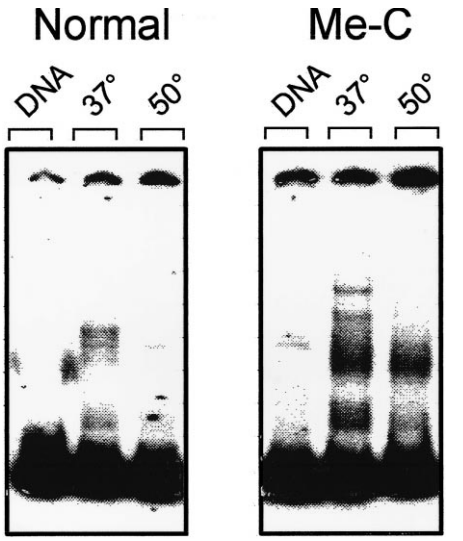


Fig. 2. Effect of temperature on the stability of the DNA-topoisomerase I complexes. The DNA (normal or Me-C) was incubated with 3 units topoisomerase I for 30 min at 37°C and then for another 30 min at 37°C or 50°C prior to gel electrophoresis. The lanes marked DNA refer to the DNA control incubated at 37°C and then 50°C (30 min each) in the absence of the enzyme.

## 3. Results and discussion

*3.1. Topoisomerase I-DNA interaction*

The binding of topoisomerase I to the *tyrT* DNA fragment was studied by a conventional gel retardation assay. The 5′-end $^{32}$P-labelled DNA fragment was incubated with human topoisomerase I for 45 min at 37°C prior to loading the samples on to a 6% non-denaturing polyacrylamide gel. The same experiments were performed in the presence of 10 μM camptothecin. The results in Fig. 1 show that the enzyme reacts differently with the DNA containing normal bases and its analogue containing 5-methylcytosine residues. The addition of the methyl group on cytosines facilitates considerably the formation of topoisomerase I-DNA complexes. Two well resolved bands (arrows in Fig. 1) can be detected with the modified DNA but not with the DNA containing canonical bases. In fact, a retarded band can be seen with normal DNA, just above the unbound DNA but this band is not well resolved compared to those detected with the modified DNA. The addition of the methyl groups which project into the major groove seems to promote the enzyme-DNA interaction. It is interesting to note that the intensity of most retarded bands is enhanced in the presence of camptothecin which freezes covalent topoisomerase I-DNA complexes.

*3.2. Stability of topoisomerase I-DNA complexes*

Topoisomerase I binds to DNA and forms transient covalent complexes via reaction of a tyrosine residue at the catalytic site with the 3′ phosphate of the cleaved strand of DNA. When the complexes formed between normal DNA and topoisomerase I at 37°C are shifted to 50°C, the complexes dissociate completely; no more retarded band is visible (Fig. 2). In contrast, with the DNA containing 5-methylcytosine residues a large fraction of the complexes is still detectable after incubating the samples for 30 min at 50°C. The addition of the methyl groups evidently increases the thermal stability of the enzyme-DNA complexes.

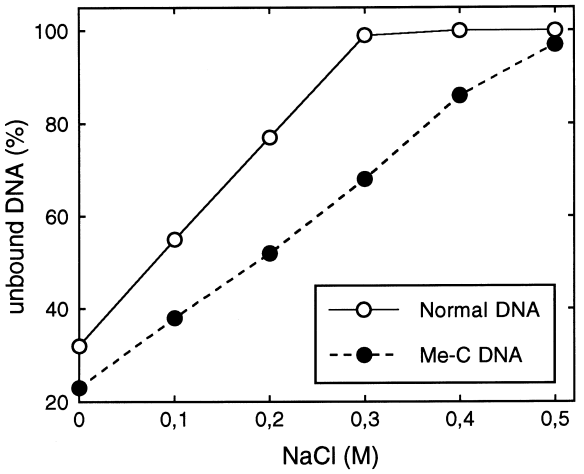


Fig. 3. Effect of NaCl on the stability of the DNA-topoisomerase I complexes. The DNA was incubated with 10 units topoisomerase I for 30 min at 37°C and then NaCl (0.05–0.5 M final concentration) was added to the samples. After 15 min incubation at 37°C, 5 μl of the loading solution was added and the products were electrophoresed on a 6% polyacrylamide gel. The intensity of the band corresponding to the unbound DNA was quantitated by densitometry.

The formation of topoisomerase I-DNA complexes is dependent upon the ionic strength of the medium. Camptothecin-stabilised cleavable complexes are rapidly reversible upon increasing the salt concentration [13]. The native gel mobility shift assay used in Figs. 1 and 2 was repeated in the presence of varying NaCl concentrations and the unbound form of DNA was quantitated by densitometry (it is more accurate to quantitate the protein-free DNA band than the smeary bands with normal DNA corresponding to the protein-DNA complexes). As shown in Fig. 3, the amount of unbound DNA was proportional to the amount of NaCl added. With both DNA species, the complexes are sensitive to the salt concentration but the effect is much more pronounced with normal DNA than with the methylated analogue. In the presence of 0.3 M NaCl, no more complexes are formed with normal DNA whereas about 30% of the material remains complexed with the DNA containing methylcytosine residues. These results also suggest that the complexes between topoisomerase I and the methylated DNA are significantly more stable than those formed with normal DNA.

### 3.3. *Sequence selectivity of camptothecin-induced DNA cleavage*

To examine more closely the effects of the incorporation of methylated cytosines, topoisomerase cleavage sites induced by camptothecin were mapped using the *tyrT* DNA. The 160 bp PCR product was uniquely end-labelled at the 5′-end of the upper strand and used as a substrate for the topoisomerase I cleavage reaction in the absence and presence of 10 μM camptothecin. The cleavage products were analysed on a sequencing polyacrylamide gel and the cleavage sites were then located and analysed by densitometry. As shown in Fig. 4, practically no bands can be detected in the absence of the inhibitor. The cleavage sites are almost imperceptible under the conditions used because the enzyme continually nicks and closes the DNA. In sharp contrast, strong bands are produced at several defined positions in the presence of camptothecin using both the methylated and non-methylated fragments. However, the patterns of topoisomerase I cleavage differ markedly for the two DNA species. Camptothecin strongly promotes DNA cleavage by human topoisomerase I at different nucleotide positions on normal and modified DNA. In other words, the addition of the methyl group provokes a major redistribution of the camptothecin-stabilised cleavage sites. A few sites such as positions 26 and 33 (5′-AT↓GA and 5′-AT↓TA) were detected with both the normal and

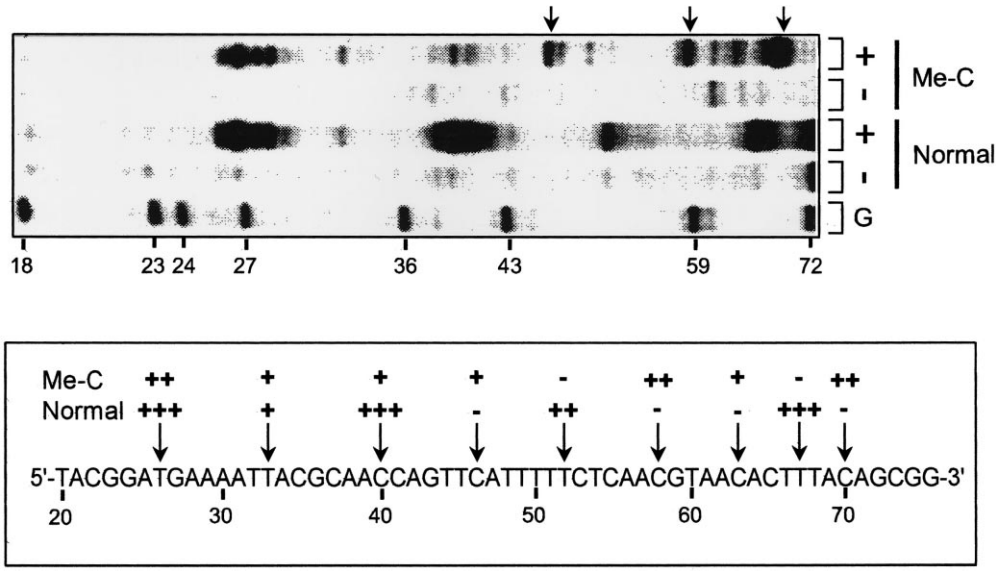


Fig. 4. Sequence analysis of the cleavage sites stimulated by camptothecin. The *tyrT* fragment containing cytosine (normal DNA) or methylcytosine residues (Me-C DNA) was incubated in the absence (−) or presence (+) of 10 μM camptothecin for 10 min prior to adding 2 μl human topoisomerase I (4 units). Reactions were allowed to proceed for 45 min at 37°C then stopped with SDS-proteinase K treatment. Topoisomerase I cleavage sites were analyzed on an 8% denaturing polyacrylamide gel. Numbers at the bottom of the gel show the nucleotide position, determined with reference to the guanine nucleotide track (G). The position of the camptothecin-stimulated cleavage sites is indicated. Symbols −, +, ++, and +++ correspond to no cleavage, weak, medium and strong cleavage, respectively. The sequence shown corresponds to that of the DNA containing natural bases. In the modified DNA, cytosines are replaced with 5-methylcytosine residues.

modified DNA. But in most cases, the cleavage sites are different. Sites at positions 52 and 67 (5′-TT↓CT and 5′-CT↓TT) are trapped by camptothecin with normal DNA but not with the methylated DNA. Conversely, sites 46, 58 and 70 (5′-TC↓AT, 5′-AC↓GT and 5′-AC↓AG, respectively) are specific to the DNA containing methylcytosines. There can be no doubt that the methyl group pointing into the major groove serves to modulate the catalytic activity of the enzyme. Previous studies have demonstrated that with normal DNA the camptothecin-induced cleavage occurs principally (but not exclusively) at sites having a T and a G on the 5′ and 3′ sides of the cleaved bond respectively [4,5]. Interestingly, the newly created sites (arrows at the top of the gel in Fig. 4) all coincide with a methylC-purine step which is analogous to a T-G step as viewed from the major groove.

## 4. Conclusion

The major groove of double helical DNA containing 5-methylcytosine residues is uniformly filled with methyl groups present on both cytosine and thymine residues. This steric hindrance is not an obstacle for topoisomerase I binding and cleavage. By contrast, the substitution of methylcytosine for cytosines facilitates the binding reaction and reinforces the stability of enzyme-DNA complexes. In addition, the C→methyl-C substitution changes the pattern of cleavage sites detected in the presence of the specific topoisomerase I inhibitor camptothecin. New cleavage sites at a defined MeC-Pu dinucleotide sites are observed. From a major groove point of view, the newly created sites at methyl-C-Pu steps resemble the T-Pu steps encountered with normal DNA. There is therefore no doubt that the methyl group exposed in the major groove of the double helix is a critical element for the interaction between topoisomerase I and DNA. This is consistent with the view that DNA methylation of cytosine at CpG sites in DNA can modulate the activity of topoisomerase I in mammalian cells [7]. It is well known that DNA methylation plays a vital role in controlling gene expression and in maintaining the integrity of the genome. These effects may be mediated, at least in part, by topoisomerase I.

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