Archives of Biochemistry and Biophysics 486 (2009) 103–110

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Conjugated eicosapentaenoic acid inhibits human topoisomerase IB with a mechanism different from camptothecin

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ARTICLE INFO

Article history:
Received 14 February 2009
and in revised form 17 April 2009
Available online 4 May 2009

Keywords:
Human DNA topoisomerase IB (hTop IB)
Conjugated eicosapentaenoic acid (cEPA)
Camptothecin (CPT)
Inhibition
Anticancer drug
Molecular docking
Autodock
Clustering

ABSTRACT

Conjugated eicosapentaenoic acid (cEPA) has been found to have antitumor effects which has been ascribed to their ability to inhibit DNA topoisomerases and DNA polymerases. We here show that cEPA inhibits the catalytic activity of human topoisomerase I, but unlike camptothecin it does not stabilize the cleavable complex, indicating a different mechanism of action. cEPA inhibits topoisomerase by impeding the catalytic cleavage of the DNA substrate as demonstrated using specific oligonucleotide substrates, and prevents the stabilization of the cleavable complex by camptothecin. Preincubation of the inhibitor with the enzyme is required to obtain complete inhibition. Molecular docking simulations indicate that the preferred cEPA binding site is proximal to the active site with the carboxylic group strongly interacting with the positively charged K443 and K587. Taken together the results indicate that cEPA inhibitor does not prevent DNA binding but inhibits DNA cleavage, binding in a region close to the topoisomerase active site.

Introduction

Topoisomerases are key enzymes that control the topological state of DNA. There are two classes of topoisomerases: type I enzymes, which act by transiently nicking one of the two DNA strands, and type II enzymes which nick both DNA strands and whose activity is dependent on the presence of ATP. These enzymes are involved in many vital cellular processes that influence DNA replication, transcription, recombination, integration, and chromosomal segregation [1–2].

A number of antitumor agents have topoisomerases as their target and they act through different mechanisms such as preventing DNA–topoisomerase binding, inhibiting the cleavage of DNA or stabilizing the topoisomerase–DNA cleavable complex [3–5]. The best characterized topoisomerase I inhibitor is camptothecin (CPT), a natural compound isolated from the bark of the Chinese tree *Camptotheca acuminata* [6]. Many derivatives of the parent compound have been synthesized and two of them, topotecan and irinotecan, have been approved by the US Food and Drug Administration for clinical use [7–9]. All the drugs belonging to the CPT family function by trapping the covalent DNA–topoisomerase complex formed by the enzyme's catalytic tyrosine and the broken DNA strand [10]. The CPT drugs bind strongly to the topoisomerase–DNA binary complex, while they do not bind to the enzyme alone and display only a weak affinity for DNA in the absence of the enzyme [11–14].

Recently it has been reported that conjugated eicosapentaenoic acid (cEPA)[1] has an inhibitory effect on human cancer cells [15]. cEPA, that in nature is found in seaweeds such as red and green algae, has been reported to have an inhibitory effect in vitro on both topoisomerase I and II and on DNA polymerases alpha and beta [16,17]. In the case of topoisomerase I cEPA was reported to inhibit supercoiled DNA relaxation [18] but so far nothing is known about the mechanism behind the inhibitory effect. In this work, we have analyzed the different steps of the catalytic cycle of topoisomerase I and showed that cEPA does not bind to the DNA–topoisomerase binary complex but interacts directly with the enzyme. The docking analysis indicates that cEPA binds in proximity of the active site, thus inhibiting DNA cleavage while still allowing the enzyme–DNA interaction.

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[1] *Abbreviations used:* cEPA, conjugated eicosapentaenoic acid; CPT, camptothecin.

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doi:10.1016/j.abb.2009.04.007

Materials and methods

cEPA synthesis. Conjugated EPA (cEPA) was prepared by alkaline treatment following the AOAC method [19] with slight modifications as described by Yonezawa and co-authors [18].

Purification of human topoisomerase IB

EKY3 cells were transformed with YEpGAL1-e-wild type, in which the human topoisomerase IB is expressed under the galactose inducible promoter in a multi-copy plasmid, as described previously [20] The protein contains an N-terminal sequence DYKDDDY recognized by the M_2 monoclonal antibodies, to be purified using an ANTI-FLAG M2 Affinity Gel column (Sigma). Elution of FLAG-fusion topoisomerase IB was performed by competition with five column volumes of a solution containing 100 μg/ml FLAG peptide in 50nM Tris–HCl, 150 mM KCl pH 7.4. Fractions were collected and glycerol was added to a final concentration of 40%; all preparations were stored at −20 °C. The fractions were resolved by SDS–polyacrylamide gel electrophoresis; protein concentration and integrity were measured through immunoblot assay, using the epitope-specific monoclonal antibody M2.

DNA relaxation assays

Topoisomerase IB was incubated in 30 μl reaction volume containing 0.5 μg of negatively supercoiled pBlueScript KSII(+) DNA and Reaction Buffer (20 mM Tris–HCl, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl, pH 7.5). To asses the effects of cEPA on enzyme activity different concentrations of the compound were added. Reactions were stopped with a final concentration of 0.5% SDS after 1 h at 37 °C. The samples were electrophoresed in a horizontal 1% agarose gel in 50 mM Tris, 45 mM boric acid, 1 mM EDTA). The gel was stained with ethidium bromide (5 μg/ml), destained with water and photographed under UV illumination. Where indicated, enzyme and inhibitor were preincubated at 37 °C for 1 min, prior of the addition of the substrate. The mixture was then incubated at 37 °C for 15 min.

The stability of the inhibitory effect of cEPA was monitored after preincubating topoisomerase I and inhibitor at 37 °C for 1 min, the inhibitor–topoisomerase mixture was then diluted in the reaction buffer, the DNA substrate was added and incubated at 37 °C for 15 min.

Assays were performed at least three times and representative gels are shown.

The effect of DNA concentration on the relaxation kinetics, in the presence or absence of cEPA (10 μM), has been examined over a range of 5–25 nM supercoiled base pairs in 20 mM Tris–HCl, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl, pH 7.5 at 13 °C. The amount of supercoiled monomer DNA band fluorescence after ethidium bromide staining was quantitated by integration using ImageQuant software. The velocities (nM DNA base pairs relaxed × min^{-1}) have been calculated in the linear region by the equation:

$$\text{Initial velocity} = \{[\text{supercoiled DNA}]_0 - (\text{Int}_t \times [\text{supercoiled DNA}]_0)/\text{Int}_0\}/t$$

where $[\text{supercoiled DNA}]_0$ is the initial concentration of supercoiled DNA, Int_0 is the area under the supercoiled DNA band at time zero, and Int_t, is the area at time t [21,22].

Cleavage/religation equilibrium assay

Oligonucleotide CL25 (5′-GAAAAAAGACTTAGAAAAATTTTTA-3′) was radiolabelled with [γ-^{32}P]ATP at its 5′ end. The CP25 complementary strand (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) was phosphorylated at its 5′ end with unlabeled ATP. The two strands were annealed at a 2-fold molar excess of CP25 over CL25 as previously described [23]. A final concentration of 20 nM duplex CL25/CP25 was incubated with an excess of topoisomerase I enzyme at 37 °C in Reaction Buffer in the presence or absence of 200 μM cEPA and/or 50 μM CPT. Dimethyl sulfoxide (DMSO) was added to no-drug controls. After 30 min the reaction was stopped by adding 0.5% SDS and digested with trypsin after ethanol precipitation. Reaction products were resolved in 20% acrylamide-7 M urea gel. The experiment was repeated at least three times and a representative gel is shown.

Kinetics of cleavage using oligonucleotide substrate

Oligonucleotide substrate CL14 (5′-GAAAAAAGACTTAG-3′) was radiolabelled with [γ-^{32}P]ATP at its 5′ end. The CP25 complementary strand (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) was phosphorylated at its 5′ end with unlabeled ATP. The two strands were annealed at a 2-fold molar excess of CP25 over CL14. The suicide cleavage reactions were carried out by incubating 20 nM of the duplex with an excess of enzyme in Reaction Buffer at 37° C and in presence of 200 μM cEPA. DMSO was added to no-drug controls. A 5 μl sample of the reaction mixture was removed before addition of the protein and used as the zero time point. At various time points 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5 μl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. Samples were analyzed by denaturing urea/polyacrylamide gel electrophoresis. The experiment was replicated at least three times and a representative gel is shown.

The percentage of cleavage at the preferential site (CL1) was quantified through PhosphoImager and ImageQuant software, comparing the percentage of the CL1 product obtained in each line to the maximal CL1 percentage obtained at the longest times.

Kinetics of religation using oligonucleotide substrate

CL14/CP25 substrate (20 nM), prepared as described above, was incubated with an excess of topoisomerase IB enzyme for 30 min at 25 °C followed by 20 min at 37 °C in reaction Buffer. A 5 μl sample of the reaction mixture was removed and used as the zero time point. Religation reactions were initiated by adding a 200-fold molar excess of R11 oligonucleotide (5′-AGAAAAATTTT-3′) over the duplex CL14/CP25 in the presence or absence of 200 μM cEPA. DMSO was added to no-drug controls. At various times 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5 μl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. Samples were analyzed by denaturing urea/polyacrylamide gel electrophoresis. The experiment was replicated three times and a representative gel is shown.

The percentage of religation was determined by PhosphoImager and ImageQuant software, normalized on the total amount of radioactivity in each lane and relatively to the highest amount of substrate converted to reaction product by human topoisomerase IB in the experiments.

Filter-binding assay

The CL25/CP25 substrate was prepared as described for the cleavage/equilibrium assay. Substrate corresponding to 20,000 cpm was incubated with increasing quantity of human DNA topoisomerase IB in presence or absence of 200 μM cEPA or 100 μM CPT, 200 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA at room temperature for 4 min in a final

volume of 100 μl. Each reaction solution was put on a pre-equilibrated cellulose acetate membrane with 100 μl salmon sperm DNA 100 μg/ml into a spin-x column (Corning 8162). After centrifugation at 6000 rpm for 1 min, the flow through was collected and the membranes were washed with 100 μl of 200 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA, 200 mM KCl; also the washing was collected. Each sample was processed for liquid scintillation photometry. The percentage of bound DNA was calculated according to the following formula:

$$\%\text{Binding} = [\text{cpm column}/(\text{cpm column} + \text{cpm flow through} + \text{cpm washing})] \times 100$$

The assay was performed at least three times.

Docking and clustering

The cEPA structure was built using the Sybyl 6.0 program (TRIPOS, http://www.tripos.com/). The topoisomerase structure (PDB ID 1A36) and the molecular dynamics (MD) trajectory refer to a previously published MD paper [24].

The MD trajectory has been clusterized, excluding DNA and linker domain, through the program g_cluster, belonging to the GROMACS 3.3.3 package [25], using the Gromos algorithm [26]. g_cluster uses each trajectory configuration as a reference, counting the configurations that show an RMSD value lower than a definite threshold. The program defines the "neighbour configurations" assigning them to a definite cluster, and the "number of neighbours" for each configuration. The reference configuration with the highest number of neighbours is defined as "representative" for the cluster and, together with its neighbours, composes a cluster. In this way all the configurations are assigned to a definite cluster, whose number depends from the threshold value.

Protein–ligand docking runs, using Lamarckian genetic algorithm [27], have been carried out, through the program Autodock 4.0 [28], for the X-ray structure (100 independent runs) and for each configuration representative of the clusters identified from the MD trajectory for a total of 600 independent runs. The grid, large 76 × 52 × 88 Å and covering the entire protein, has been centered close to the protein geometric center.

The complexes have been inspected through an in house modified version the g_mindist program, belonging to the GROMACS 3.3.3 package [25], in order to select the protein atoms with a distance lower than 3.5 Å from any cEPA atom and so identify the most frequently sampled cEPA–topoisomerase complex.

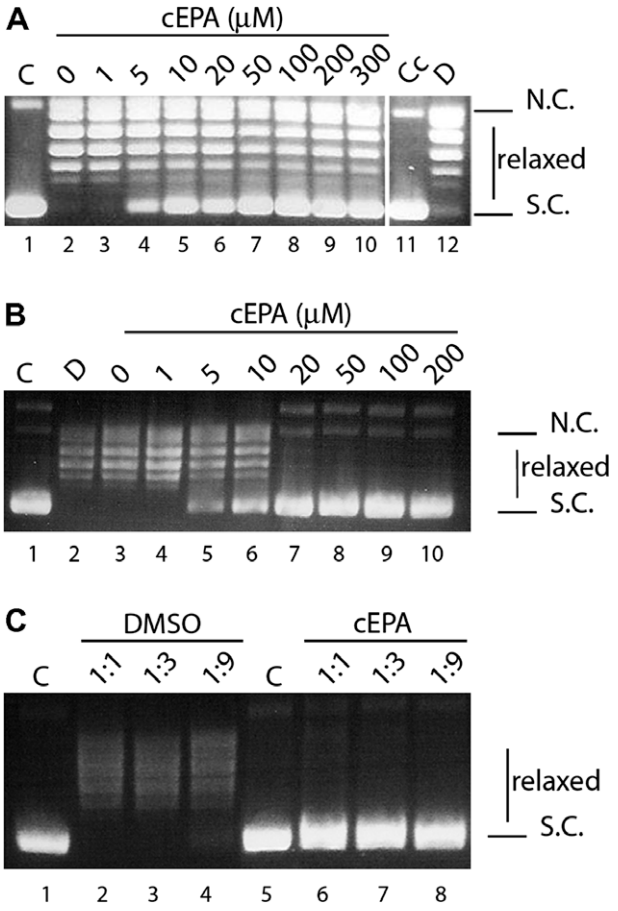

Fig. 1. (A) Relaxation of negative supercoiled plasmid DNA by topoisomerase IB in the presence of increasing concentrations of cEPA (lanes 2–10). The reaction products were resolved in an agarose gel and visualized with ethidium bromide. Lane 1, no protein added. Lane 11, control reaction with only substrate and cEPA (300 μM). Lane 12, control reaction with DNA, enzyme and DMSO, in the absence of cEPA. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA. (B) Relaxation assay of topoisomerase IB preincubated with increasing concentration of cEPA for 1 min at 37 °C, before the addition of substrate (lanes 3–10). Lane 1, no protein added. Lane 2, control reaction with DNA, enzyme and DMSO, in the absence of cEPA. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA. The band that migrates slower than the nicked circular DNA consists of dimers of the supercoiled plasmid DNA that also become relaxed. (C) Relaxation assay of topoisomerase IB preincubated with only DMSO or cEPA (200 μM) for 1 min and then diluted 3- or 9-fold. Lanes 1 and 5, no protein added. SC, supercoiled plasmid DNA.

Results and discussion

Effect of cEPA on topoisomerase IB activity

The inhibitory effect of cEPA (conjugated eicosapentaenoic acid) on the human topoisomerase I activity, determined by a plasmid relaxation assay, is shown in Fig. 1A. The assay detects the different electrophoretic mobility of the DNA supercoiled plasmid converted, by the enzyme, to its relaxed form, in presence of increasing concentrations of the compound. The data indicate that cEPA inhibits the human topoisomerase I in a dose dependent manner (Fig. 1A, lane 2–10) and the maximal inhibition of the enzyme activity is achieved at 50 μM concentration, although a complete inhibition is never reached under this conditions. As a control it is shown that cEPA does not effects the electrophoretic mobility of DNA in the absence of topoisomerase I (Fig. 1A, lane 11). Since cEPA is dissolved in DMSO, an assay of the enzyme in presence of an identical concentration of DMSO without cEPA has also been carried out, to show that it has no effect on the relaxation activity of topoisomerase I (Fig. 1A, lane 12).

The plasmid relaxation assay, carried out after preincubating the enzyme with increasing concentrations of cEPA before the addition of DNA, shows a greater inhibitory effect on topoisomerase activity (Fig. 1B). The DNA relaxation is completely inhibited at 100 μM cEPA (Fig. 1B, lane 8). The complete inhibition also remains after a 3- or 9-fold dilution of the preincubated cEPA–enzyme mixture, indicating that the inhibitory effect is irreversible (Fig. 1C, lanes 6–8). As a control it is shown that the enzyme only preincubated with DMSO in the absence of cEPA and diluted as the sample maintains its activity (Fig. 1C, lanes 2–4).

Effects of cEPA on cleavage/religation equilibrium

In the case of CPT the inhibition mechanism is well characterized, the drug reversibly binds to the covalent intermediate DNA–enzyme, stabilizing the cleavable complex and reducing the religation rate [13]. For cEPA the mechanism is still unknown, and to clarify it a series of experiments have been carried out using an excess of cEPA, namely 200 μM. To understand the effects of

cEPA on the stability of the covalent DNA–enzyme complex a cleavage/religation equilibrium experiment on the 25 mer full duplex oligonucleotide substrate CL25 (5′-GAAAAAAGACTTAGAA AAATTTTTA-3′)/CP25 has been carried out in the presence of cEPA, CPT or cEPA and CPT together. After 30 min of incubation the reactions were stopped with SDS, the samples digested with trypsin and the products analyzed by polyacrylamide–urea gel electrophoresis (Fig. 2). In absence of inhibitors (Fig. 2, lane 2) the cleavage/religation equilibrium is shifted toward religation, with no detectable trapped cleavable complex. The same is seen in presence of 200 μM cEPA (Fig. 2, lane 3). On the other hand when the enzyme is incubated with 50 μM CPT the cleavage/religation equilibrium is shifted toward cleavage, since the band corresponding to the substrate cleaved at the preferred CL1 site is clearly detectable (Fig. 2, lane 4). Preincubation of cEPA with the enzyme before addition of duplex oligonucleotide and CPT (Fig. 2, lane 5) does not permit the stabilization of the cleavable complex by CPT. These data indicate that either cEPA inhibits the cleavage or induces a faster religation, so that the cleavable complex cannot be formed or cannot be stabilized by CPT.

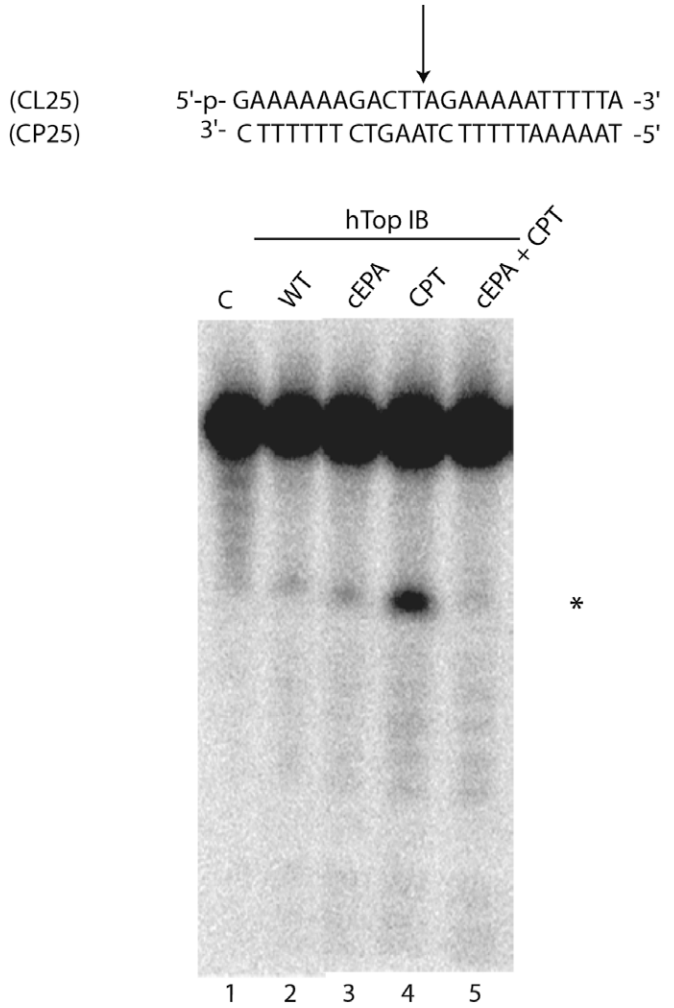

Fig. 2. Cleavage/religation equilibrium of the duplex substrate, shown at the top of the figure, containing 5′ end-labeled CL25 and the preferred topoisomerase I binding site indicated by an arrow. The duplex is incubated with the enzyme alone (lane 2) or in presence of cEPA (lane 3), or CPT (lane 4). Lane 1, no protein added. Lane 5, topoisomerase I preincubed with cEPA before the addition of DNA and CPT. The cleavage products were analyzed in a urea–polyacrylamide gel. The slowest migrating band corresponds to the uncleaved oligonucleotide. The asterisk indicates the band corresponding to the preferential cleavage site.

Analysis of the religation rate

To understand whether cEPA affects the cleavage/religation equilibrium perturbing the religation or the cleavage reaction, these two processes were evaluated in separate experiments. To measure the religation rate the oligonucleotide substrate CL14 (5′-GAAAAAAGACTTAG-3′), 5′ end radiolabeled and containing a preferred cleavage site for the enzyme [23], has been annealed to the CP25 (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) complementary strand to produce the suicide substrate, i.e. a duplex with an 11-base 5′ single strand extension. With this substrate the enzyme is not able to carry out the religation step, because the dinucleotide, generated during cleavage, cannot be religated [29]. In order to measure the religation rate the suicide cleavage substrate has been incubated with an excess of native enzyme to allow suicide cleavage to proceed to completion, then a 200-fold molar excess of the complementary R11 oligonucleotide (5′-AGAAAAATTTT-3′) has been added in presence or absence of 200 μM cEPA inhibitor. The urea–polyacrylamide gel of different aliquots, analyzed as a function of time, is reported in Fig. 3A. An identical religation rate is observed independently of the presence of cEPA, as shown by the plot of the percentage of the relegation product reported in Fig. 3B. The opposite is found in the presence of CPT (data not shown), which is known to strongly reduce the topoisomerase IB religation

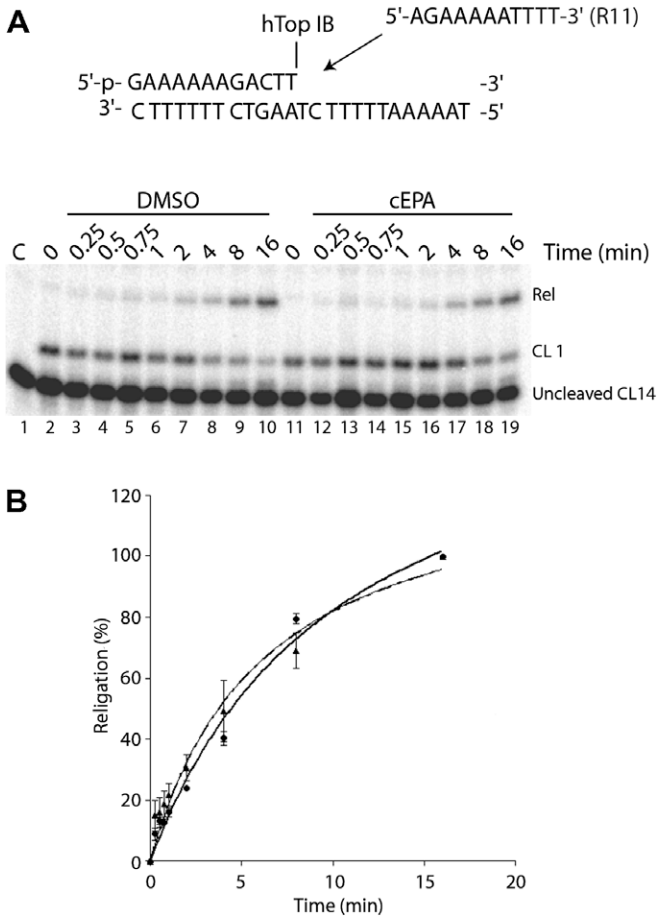

Fig. 3. (A) Gel analysis of the religation kinetics for topoisomerase IB in absence (lanes 2–10) or presence of cEPA (lanes 11–19) with the R11 substrate and the topoisomerase IB suicide substrate covalent complex, shown at the top of the figure. CL1, represents the DNA fragment cleaved at the preferred enzyme site. Rel represents the religation product. (B) Percentage of the religation product plotted at different times for topoisomerase IB in absence (circles) or presence of cEPA 200 μM (triangles). The data reported are the average ± SD of three independent experiments.

rate [30,14]. Therefore cEPA must have an inhibition mechanism different from that of CPT.

Analysis of the cleavage rate

The cleaved DNA fragments deriving from a time course experiment of a suicide cleavage substrate incubated with topoisomerase I are shown in Fig. 4A. The cleavage reaction is very fast for the enzyme in absence of the inhibitor. In fact, 85% of the cleavage product (quantified and normalized to the total amount of radioactivity in each lane) is produced in 15 s and after 2 min the maximum quantity of cleaved substrate is reached (Fig. 4B, circle). In the presence of cEPA the cleavage reaction is inhibited; the band corresponding to the cleaved substrate is quite weak and not more than ~40% of the product can be obtained even after long times (Fig. 4B, triangle).

Cleavage inhibition is enhanced when the enzyme is preincubated with cEPA (Fig. 4A and B). The experiments indicate that, upon preincubation, the conjugated fatty acid fully inhibits the catalytic cleavage activity of the topoisomerase IB, but these results do not clarify if this is due to prevention of the DNA–enzyme binding or to inhibition of the catalytic reaction.

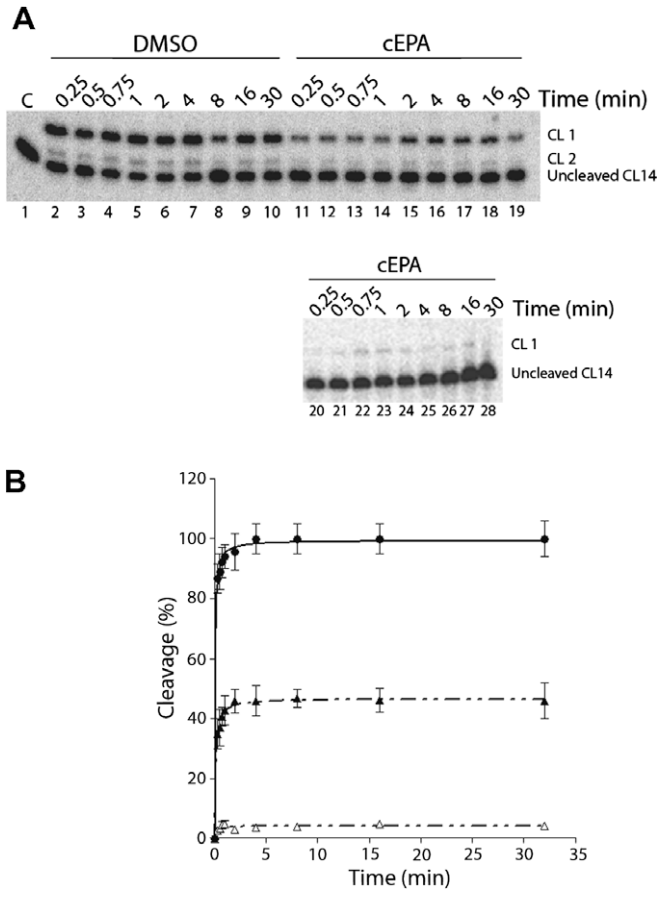

Fig. 4. (A) Suicide cleavage kinetics with the CL14/CP25 substrate for topoisomerase IB in absence (lanes 2–10), in presence of 200 μM cEPA (lanes 11–19), or after preincubation with cEPA (lanes 20–28). Lane 1, no protein added. CL1 represents the DNA fragment cleaved at the preferred enzyme site; CL2 represents a second cleavage site cleaved by the enzyme, but to a lower extent that CL1. (B) Percentage of the CL1 product, quantified as described in Materials and methods, in the absence (circles) and presence (triangles) cEPA and after preincubation with 200 μM cEPA for 1 min at 37 °C (open triangles). The data are average ± SD of three independent experiments.

Filter-binding assay for topoisomerase IB

Yonezawa and co-authors have already demonstrated that cEPA does not bind to double strand DNA alone, since the melting temperature of double strand DNA does not change in the presence of the inhibitor [18]. A filter-binding assay has been then carried out on the enzyme–DNA mixture in the presence or absence of cEPA to determine the role of this compound in modulating the DNA-enzyme interaction. The protein–DNA binding was followed using a radiolabeled 25 mer full duplex oligonucleotides substrate CL25/CP25, shown at the top of the Fig. 2. The oligo has been incubated with increasing amounts of enzyme alone or in presence of cEPA (200 μM) or CPT (100 μM) on a nitrocellulose filter, maintaining a constant percentage of glycerol. The CPT has been used as a positive control since it traps the cleavable complex and should then give rise to a large amount of trapped DNA–enzyme complex [31].

The plot of the percentage of binding reported in Fig. 5 shows that there is no difference in the DNA binding ability for the topoisomerase in the presence or absence of cEPA. These data demonstrate that the cEPA inhibitor does not prevent the enzyme–DNA binding although it does inhibit the formation of the cleavable complex (Fig. 4). In presence of CPT an higher percentage of DNA–enzyme complex is observed (Fig. 5) confirming that CPT and cEPA inhibit the enzyme with different mechanisms: CPT stabilizes the cleavable complex strongly and slows down the religation process [32], while cEPA interacts with the enzyme without preventing its interaction with DNA, but inhibiting the cleavage of the DNA strand, likely through an uncompetitive mechanism.

Analysis of the cEPA inhibitory mechanism

The cEPA inhibitory mechanism has been monitored following the topoisomerase relaxation activity as a function of substrate concentration in absence or in presence of 10 mM cEPA. The relaxation assay has been carried out at 13 °C to slow down the reaction and better detect the produced DNA topoisomers. The corresponding Lineweaver–Burk plot (Supplementary Fig S1) showing 1/V vs 1/S indicates that the data can be interpolated by a straight line intercepting, either in presence or in absence of cEPA, the *x*-axis on the negative side at the same value, confirming that cEPA is inhibiting the enzyme with an uncompetitive mechanism.

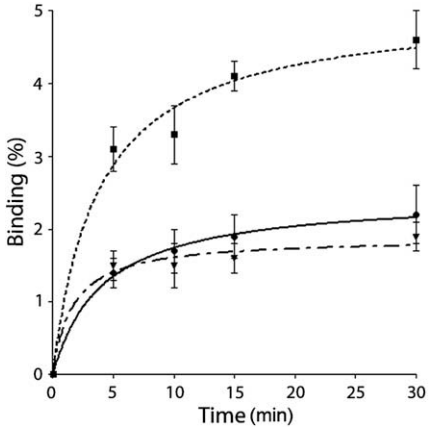

Fig. 5. Percentage of DNA–topoisomerase IB binding as a function of protein concentration. The assay has been performed with the enzyme alone (circles), in the presence of 100 μM CPT (squares) or 200 μM cEPA (triangles). The data are an average of three independent experiments. The error bars indicate the SD.

Docking and clustering

Trajectory clustering, carried out on a topoisomerase MD trajectory recently made in our laboratory [23], identifies, using a threshold of 1.5 Å, 17 protein clusters. The first six clusters, that cover 93.5% of the total trajectory, have been selected for the molecular docking simulations. A total of 700 protein–ligand runs have been carried out, 100 for the X-ray structure and 100 for each configuration representative of the six protein clusters.

In order to identify the preferred cEPA binding site, the percentage of each protein residue having a distance lower than 3.5 Å from the cEPA carboxyl group, has been calculated for the 700 complexes. The results indicate that the preferred contacted residues are: K443, K587 and N722, in 28%, 29% and 26% of the complexes, respectively. Moreover K443, K587 and N722 are simultaneously contacted (distance lower than 3.5 Å) by the carboxylic group in 23% of the complexes (i.e. 160 complexes). This result indicates that this is the preferred cEPA binding site and provides a molecular explanation for the inhibitory effect of cEPA on the cleavage reaction. The cEPA acid, in fact, interacting at once with K443, K587 and N722, close to the active site residues (see Fig. 6), prevents the catalytic Y723 to execute the nucleophilic attack on the DNA phosphate.

It is interesting to notice that whilst the carboxyl group shows a preferred orientation, the hydrophobic tail of cEPA branch out over the protein surface and is arranged in many different hydrophobic clefts. These 160 complexes have been clusterized, using a threshold of 5 Å, obtaining 11 different families, whose representative conformations are displayed in Fig. 6. This large number of observed cEPA conformations are stabilized by hydrophobic contacts established in crevices that extend from the carboxylic binding site, everyone equally interacting with the hydrophobic cEPA chain. Actually all these 11 complexes are characterized by a free energy value ranging from −7.7 to −4.8 kcal/mol, confirming their similar stability and providing a thermodynamic explanation for the not preferred orientation of the hydrophobic cEPA chain. The steric hindrance, generated by each of these cEPA conformations, is not large enough to prevent the DNA binding as shown in Fig. 7 where the topoisomerase–cEPA–DNA ternary complex is represented. This finding is in agreement with the experimental results, indicating the inhibition of the cleavage reaction (Fig. 2) and the maintenance of the DNA binding properties (Fig 5).

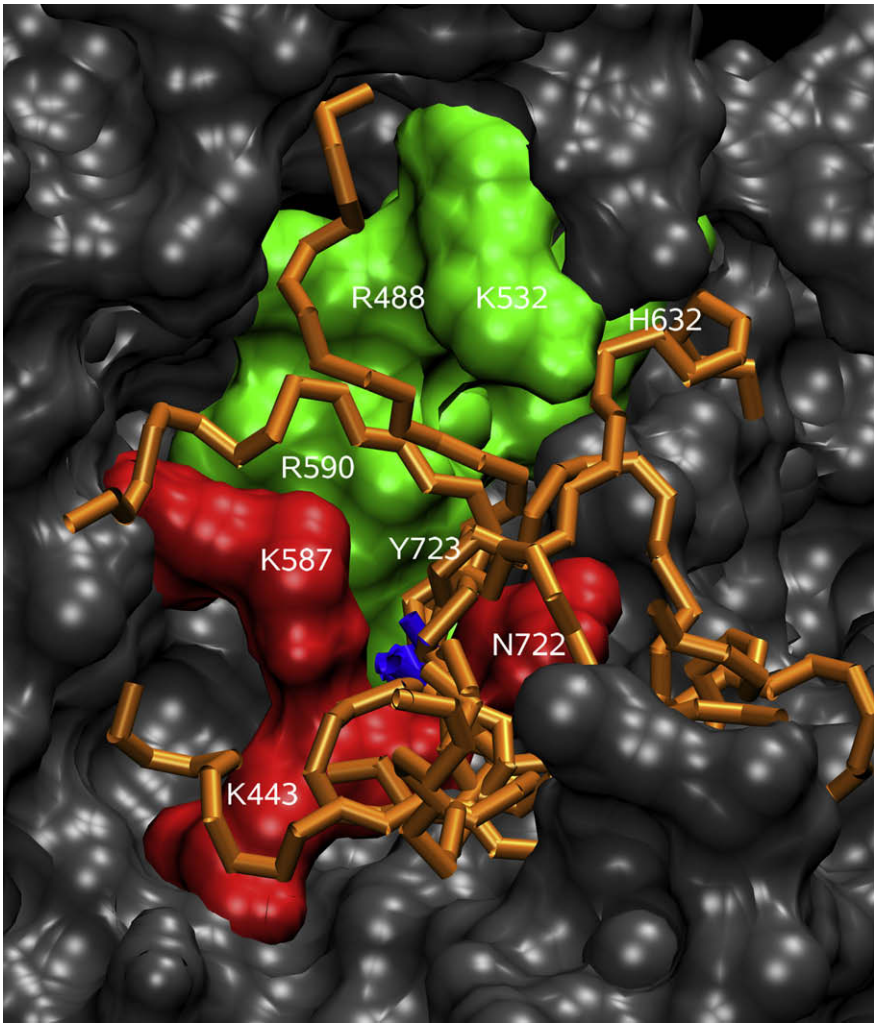

Fig. 6. Representation of the solvent accessible surface enzyme region proximal to the active site with the 11 cEPA conformations, representative of the 11 clusters obtained from the 160 best cEPA–enzyme complexes. cEPA is depicted as a stick model. The cEPA carboxylic groups is represented in blue, the hydrophobic chain in orange, the contacted residues K443, K587 and N722 in red ant the catalytic pentad (R488, K532, R590, H632, Y723) in green. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

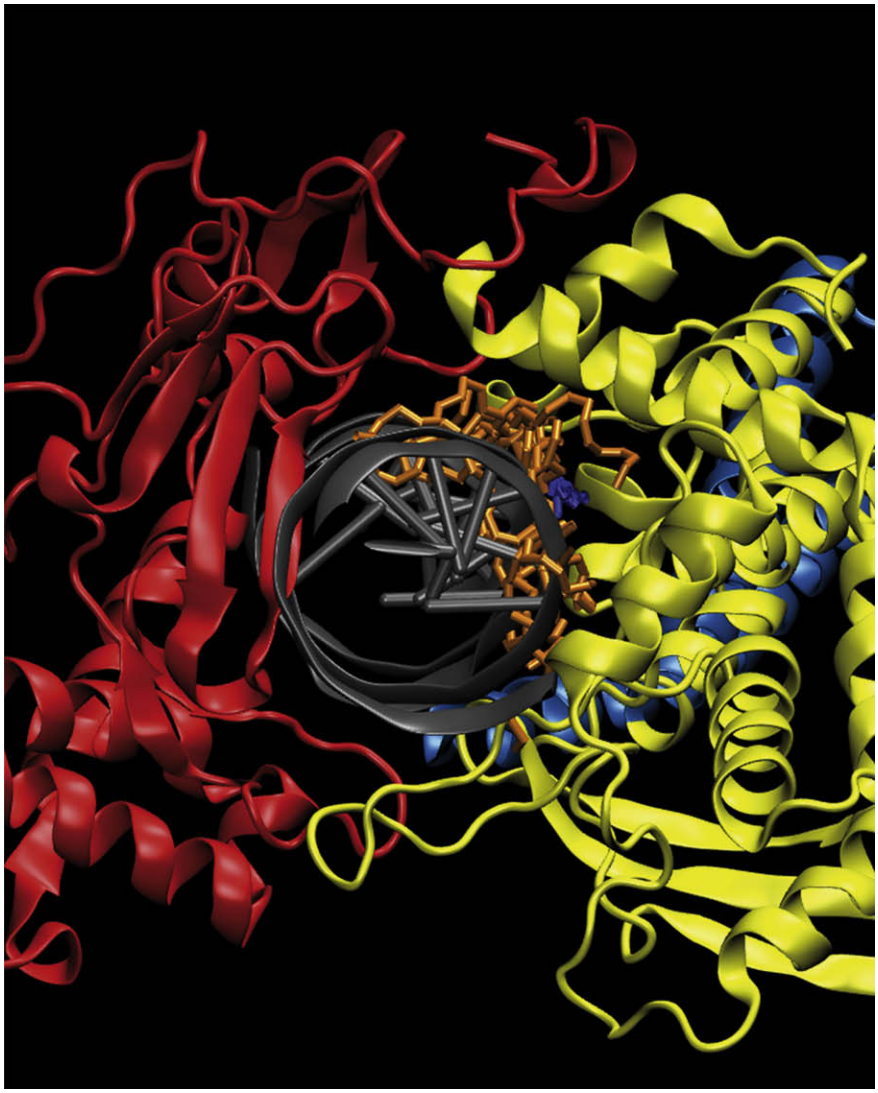

Fig. 7. Representation of the 11 cEPA conformations on the enzyme–DNA binary complex. Protein subdomain I and subdomain II (red), subdomain III and C-terminal domain (yellow), linker domain (light blue) and DNA (gray) are depicted as ribbon, cEPA is depicted as a stick model with the carboxylic group in blue and the hydrophobic chain in orange. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

Conclusion

The plasmid relaxation assays demonstrate that cEPA is a topoisomerase I inhibitor (Fig. 1A) able to fully inhibits the enzyme after preincubation (Fig. 1B). The inhibition is irreversible since dilution of the cEPA–enzyme mixture does not restore any activity. (Fig. 1C). The cEPA inhibitory mechanism is different from CPT, as demonstrated by cleavage, religation and DNA-binding experiments. cEPA does not perturb the cleavage/religation equilibrium observed with the enzyme alone (Fig. 2) at variance of CPT, which shifts the equilibrium toward cleavage. Moreover preincubation with cEPA prevents the formation of the ternary complex stabilized by CPT (Fig. 2). cEPA does not effect the religation rate of topoisomerase I (Fig. 3) at variance on what observed in presence of CPT [32]. On the other hand cEPA inhibits cleavage (Fig. 4A) and this effect is enhanced by preincubation (Fig. 4B). Moreover the inhibition of the enzyme with cEPA does not affect the DNA substrate binding (Fig. 5) suggesting uncompetitive nature of the inhibition. In contrast CPT does not bind either topoisomerase I or DNA individually but does bind and stabilize the enzyme–DNA covalent intermediate [33]. The docking of the cEPA molecule to topoisomerase provides a clear molecular explanation for such experimental results. In fact the docking procedure indicate that a stable cEPA–topoisomerase complex is obtained when its carboxylic group sits on the active site establishing a strong electrostatic interaction with K773 and K587, whilst the aliphatic chain accommodates itself in several crevices close to the active sites. The formation of this cEPA–topoisomerase binary complex does not prevent DNA binding since cEPA sits on the active site not occupying the volume used by the DNA substrate to interact with the enzyme (Fig. 7) in perfect agreement with the experimental results.

In conclusion we have characterized at functional and structural level the interaction of cEPA with topoisomerase IB demonstrating that it inhibits the cleavage reaction binding in proximity of the active site and leaving enough volume to permit the binding of the DNA substrate.

Acknowledgments

This work was supported by AIRC grant "Characterization of human topoisomerase I mutants resistant to camptothecin and its derivatives" to A.D. and by a fellowship from AIRC/FIRC to S.C. We thank S.Z. Pedersen for carefully reading this article and for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2009.04.007.

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