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Erybraedin C, a natural compound from the plant *Bituminaria bituminosa*, inhibits both the cleavage and religation activities of human topoisomerase I

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The interaction of human topoisomerase I and erybraedin C, a pterocarpan purified from the plant *Bituminaria bituminosa*, that was shown to have an antitumour activity, was investigated through enzymatic activity assays and molecular docking procedures. Erybraedin C is able to inhibit both the cleavage and the religation steps of the enzyme reaction. In both cases, pre-incubation of the drug with the enzyme is required to produce a complete inhibition. Molecular docking simulations indicate that, when interacting with the enzyme alone, the preferential drug-binding site is localized in proximity to the active Tyr^{723} residue, with one of the two prenilic groups close to the active-site residues Arg^{488} and His^{632}, essential for the catalytic reaction. When interacting with the cleavable complex, erybraedin C interacts with both the enzyme and DNA in a way similar to that found for topotecan. This is the first example of a natural compound able to act on both the cleavage and religation reaction of human topoisomerase I.

Key words: erybraedin C (ERYC), human topoisomerase I, inhibition mechanism, inhibitor, molecular docking, pterocarpan.

INTRODUCTION

DNA topoisomerases are key enzymes that modulate the topological state of DNA through the breaking and rejoining of DNA strands. There are two classes of topoisomerases (type I and type II), both characterized by a catalytic mechanism which involves a nucleophilic attack of a DNA phosphodiester bond by a tyrosyl residue from the enzyme, but type I cleaves one DNA strand, whereas type II cleaves both strands. These enzymes have been shown to be essential in nearly all processes of DNA metabolism such as replication, transcription, recombination and chromosomal segregation [1,2]. The human Top1 (topoisomerase I) enzyme is composed of 765 amino acids and has four distinct domains: the N-terminal domain (1–214), the core domain (215–635), the linker domain (636–712) and the C-terminal domain (713–765) [3–5]. The three-dimensional structure of the reconstituted N-terminal truncated version of human Top1 in complex with a 22 bp DNA molecule shows the enzyme organized in multiple domains which 'clamp on' the DNA molecule [4]. The active site tyrosine (Tyr^{723}) starts the catalytic cycle of the enzyme through a nucleophilic attack on the DNA backbone, resulting in the breakage of one DNA strand with the enzyme covalently attached to the 3′-phosphate to form the cleavable complex. After changing the linking number, a second nucleophilic attack, driven by the 5′-hydroxy DNA end, restores an intact double-stranded DNA, and the enzyme is released [5].

DNA topoisomerase IB can be inhibited by several compounds that act through different mechanisms such as prevention of DNA-topoisomerase binding, inhibition of DNA cleavage or stabilization of the cleavable complex. Inhibitors of Top1 are divided into two classes: poisons and catalytic inhibitors [6,7]. Poisons include clinically used drugs, such as the derivatives of the natural compound CPT (camptothecin) that reversibly bind the covalent Top1–DNA complex, slowing down the re-ligation of the cleaved DNA strand, thus inducing cell death. Two water-soluble CPT derivatives, TPT (topotecan) and irinotecan, have been approved by the FDA for clinical use. Catalytic inhibitors act through inhibiting any other step of the Top1 enzymatic cycle [8].

Natural antioxidants from plants, such as pterocarpans, the second group of isoflavones, have currently received interest because of their antimicotic, antiviral and antitumoral activity [9]. The pterocarpan ERYC (erybraedin C) has previously been purified from the flower aerial parts of *Bituminaria bituminosa* and *Bituminaria morisiana* and was chemically characterized [10,11]. This drug contains a tetracyclic ring system derived from the basic isoflavonoid skeleton by an ether linkage between position 4 and 2 and is characterized by the presence of two hydroxy groups, located, respectively, on the 7 and 4′ position, and two prenyl groups (γ,γ-dimethylallyl) on the 8 and 5′ position (Figure 1). Because of the presence and distribution of phenolic hydroxy and prenyl substituents, ERYC may exhibit a high chain-breaking antioxidant potential [12,13]. In addition, ERYC exhibits anticlastogenic activity against mytomicin and the radio-mimetic bleomycin [14]. ERYC induced apoptosis in two human adenocarcinoma cell lines (HT29 and LoVo) proficient and deficient in MMR (mismatch repair), p53 and Bcl-2 with a pattern that was proposed to proceed through topoisomerase II poisoning [9].

In the present study we have investigated for the first time the interaction of ERYC with Top1 enzyme, demonstrating that the drug inhibits both the cleavage and the religation processes. Molecular docking indicates that ERYC, when interacting with the enzyme alone, binds in the proximity of the active site,

Abbreviations used: CPT, camptothecin; EMSA, electrophoretic mobility-shift assay; ERYC, erybraedin C; SC-uracil, synthetic complete medium lacking uracil; Top1, topoisomerase I; TPT, topotecan.

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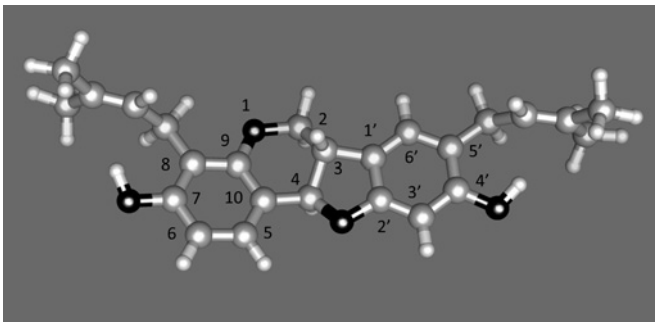

Figure 1 Molecular structure of ERYC

Ball and stick representation of the ERYC molecule with the C, O and H atoms coloured grey, black and white respectively. The numeration of the atoms follows that described by Maurich et al. [14].

blocking the catalytic reactions but still permitting the binding of the DNA substrate. Docking of ERYC with the protein–DNA cleavable complex indicates that the drug intercalates the DNA bases in a way similar to that found for TPT.

EXPERIMENTAL

Yeast strains, plasmids and chemicals

CPT (Sigma) and ERYC (provided by G. Turchi) were dissolved in DMSO to a final concentration of 4 mg/ml and 4.5 mg/ml respectively and stored at −20 °C. Anti-FLAG M2 affinity gel, FLAG peptide and M2 monoclonal antibody were purchased from Sigma. *Saccharomyces cerevisiae* strain EKY3 (ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1::TRP1, MATα) was previously described in [15]. Plasmid YCpGAL1 wild-type and the catalytically inactive mutant Y723F were as described previously [16,17]. The epitope-tagged constructs YCpGAL1-e-wild-type and the YCpGAL1-e-Y723F contain the N-terminal sequence FLAG: DYKDDDY (indicated with 'e'), recognized by the M2 monoclonal antibody.

Purification of DNA Top1

EKY3 cells were transformed with YCpGAL1-e-wild-type or YCpGAL1-e-Y723F, grown on SC-uracil (synthetic complete medium lacking uracil) plus 2 % (w/v) dextrose and diluted 1:100 in SC-uracil plus 2 % (v/v) raffinose. At $A_{595} = 1.0$, the cells were induced with 2 % (v/v) galactose for 6 h. Cells were then harvested by centrifugation, washed with cold water and resuspended in 2 ml of buffer/g of cells using a buffer containing 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 % (v/v) glycerol, complete protease inhibitor cocktail (Roche 1836153) and supplemented with 0.1 mg/ml sodium bisulfite and 0.8 mg/ml sodium fluoride. After addition of 0.5 vol. of 425–600 μm diameter glass beads, the cells were disrupted by vortexing for 30 s alternating with 30 s on ice. The lysate was centrifuged and a final concentration of 0.15 M KCl was added to the sample prior to loading on to a 2 ml anti-FLAG M2 Affinity Gel column equilibrated as described by the manufacturer (Sigma). The column was washed with 20 column volumes of TBS (50 mM Tris/HCl and 150 mM KCl, pH 7.4). Elution of FLAG-tagged Top1 was performed by competition with five column volumes of a solution containing 100 μg/ml FLAG peptide in TBS (Tris-buffered saline). Fractions of 500 μl were collected and a final concentration of 40 % (v/v) glycerol was added; all preparations were stored at −20 °C. The fractions were resolved by SDS/PAGE; protein concentration and integrity were measured through immunoblot assay using the epitope-specific monoclonal antibody M2.

DNA Top1 activity *in vitro*

Top1 activity was assayed with a DNA relaxation assay. Top1 preparations were incubated in a 30 μl reaction volume containing 0.5 μg of negatively supercoiled plasmid pBlueScript KSII(+)DNA and reaction buffer (20 mM Tris/HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl).

To assess the effects of ERYC or CPT on enzyme activity, different concentrations of the compounds were added. All of the assays have been carried out in the presence of 150 mM KCl, since in its absence the enzyme does not release the substrate. Reactions were stopped with a final concentration of 0.5 % SDS after 1 h at 37 °C and electrophoresis of the samples was carried out in a 1 % (w/v) agarose gel in TBE1 (96 mM Tris, 91 mM boric acid and 1.9 mM EDTA) at 10 V/cm. Where indicated, enzyme and inhibitor were pre-incubated at 37 °C for 10 min, prior to the addition of the substrate. The mixture was then incubated at 37 °C for 15 min. The stability of the inhibitory effect of ERYC was monitored after pre-incubating Top1 and inhibitor at 37 °C for 10 min, the inhibitor–Top1 mixture was then diluted in the reaction buffer, then the DNA substrate was added and incubated at 37 °C for 15 min.

Cleavage/religation equilibrium

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 unlabelled ATP. The two strands were annealed at a 2-fold molar excess of CP25 over CL25. A final concentration of 20 nM duplex CL25/CP25 was incubated with an excess of enzyme at 25 °C in 20 mM Tris/HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$ and 50 μg/ml acetylated BSA in the presence or absence of 50 μM CPT or 100 μM ERYC. After 30 min, the reaction was stopped by adding 0.5 % SDS and digested with trypsin after ethanol precipitation. Reaction products were resolved in 16 % (v/v) acrylamide/7 M urea gels in TBE2 (48 mM Tris, 45.5 mM boric acid, 1 mM EDTA).

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 unlabelled ATP. CL14/CP25 was annealed as previously described. The suicide cleavage reactions were carried out by incubating 20 nM of the duplex with an excess of enzyme pre-incubated with DMSO or different concentrations of ERYC for 10 min, in 20 mM Tris/HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl at 23 °C in a final volume of 50 μl as described by Yang and Champoux [18]. After 30 min, 5 μl aliquots were removed and the reaction was 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 analysed by denaturing urea/polyacrylamide gel electrophoresis in TBE2 (48 mM Tris, 45.5 mM boric acid and 1 mM EDTA).

Kinetics of religation using oligonucleotide substrate

CL14/CP25 (20 nM) was incubated with an excess of enzyme for 60 min at 23 °C followed by 30 min at 37 °C in 20 mM Tris/HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl. The cleavable complex formed was pre-incubated for 10 min with DMSO, as a control, or ERYC. A 5 μl sample of the reaction mixture was removed before addition of R11 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. At 37 °C 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 analysed by denaturing urea/polyacrylamide gel electrophoresis in TBE2 (48 mM Tris, 45.5 mM boric acid, 1 mM EDTA).

EMSA (electrophoretic mobility-shift assay)

The double-stranded 25 bp oligonucleotide CL25/CP25 was prepared as described for the cleavage/equilibrium assay. The reactions were carried out using the catalytically inactive mutant enzyme Y723F. Two different concentrations of mutant enzyme were incubated in standard reaction conditions [20 mM Tris/HCl, pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated BSA and 150 mM KCl] in the presence of 1 % (v/v) DMSO or 100 μM ERYC at 37 °C for 10 min in a final volume of 30 μl. After pre-incubation, DNA substrate was added and the binding reactions were performed for 10 min at 37 °C. Reactions were stopped by the addition of 7 μl of dye [0.125 % Bromophenol Blue and 40 % (v/v) glycerol]. Samples were loaded on to 6 % (v/v) native polyacrilamide gels and electrophoresed at 70 V in TBE3 (12 mM Tris, 11.4 mM boric acid and 0.2 mM EDTA). Products were visualized by phosphorimaging.

Molecular Docking procedure

Molecular Docking is a computational technique that predicts the putative binding site of a ligand on a receptor, taking into account both geometrical and electrostatic match contributions. The Docking was conducted with the Autodock 4 program, using the AutodockTools suite version 4 to prepare the structures of ligand and receptor [19]. The Docking runs with the free enzyme have been carried out using the structure of the protein from the crystal structures 1A36 [5] and 1EJ9 [20], where missing residues were reconstructed with a procedure described previously [21,22], once the DNA substrate was eliminated. The docking with the binary complex was carried out using the three-dimensional co-ordinates from the ternary complex crystal structure 1K4T [23], after elimination of TPT. Partial atomic charges of ERYC were calculated using the RESP (Restrained Electrostatic Potential) fitting method [24]. Geometric optimization was carried out with Gaussian03 [25] at B3LYP/6-31G* level of theory. The water solvation effect was taken into account by means of the COSMO variant of the polarizable continuum model [26]. For the docking experiment, 250 runs were performed, using the Lamarckian Genetic Algorithm [27]. The simulative box [$38 \times 48 \times 38$ Å (1 Å=0.1 nm)] was built to contain the inner cavity of the protein, dedicated to accommodate the DNA, and the residues lateral chains protruding into it, and centred on its geometric centre. The analysis of the contacts between the ligand and the receptor in all the resulting structures was performed using a modified version of the program g_mindist from the Gromacs 3.3.3 package [28], taking a threshold value of 3.5 Å. Images were obtained with the UCSF Chimera [29] and VMD visualization packages [30].

RESULTS

ERYC inhibits the catalytic activity of human Top1

The inhibitory effect of ERYC on human Top1 activity was assessed by a plasmid relaxation assay (Figure 2A). Native protein was incubated with a supercoiled plasmid in the absence or presence of an increasing concentration of ERYC and the relaxation activity was monitored after 1 h. The results indicate that ERYC inhibits the relaxation activity of Top1 in a dose-dependent manner and also as a function of the pre-incubation with the enzyme (Figure 2A). In fact, simultaneous addition of enzyme, ERYC and DNA determines an inhibition of the relaxation activity that starts at 8 μM ERYC (Figure 2A, lane 4) and is maximal at a drug concentration of 20–25 μM (Figure 2A, lanes 7 and 8), although a complete inhibition is never achieved under these conditions. The assay, carried out after pre-incubating the enzyme with increasing concentrations of ERYC before the addition of DNA, shows a greater inhibitory effect on Top1 activity, with a full inhibition at 12 μM concentration (Figure 2A, compare lanes 5 and 13). As a control, it is shown that ERYC does not affect the electrophoretic mobility of DNA in the absence of Top1 (Figure 2A, lane 17). Since ERYC is dissolved in DMSO, an assay of the enzyme activity in the presence of an identical concentration of DMSO without ERYC was also carried out, to show that DMSO does not affect the relaxation activity of Top1 (Figure 2A, lanes 1 and 9). This result indicates that, upon incubation, ERYC is an efficient inhibitor of the relaxation, more efficient than CPT when used at the same concentration. In fact, when the relaxation is carried out after 1 h, a slight inhibitory effect of CPT is observed only at 20/25 μM concentration (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/425/bj4250531add.htm). This could be in part expected, since CPT is known to be a reversible inhibitor [31]. The assay was then carried out as a function of time for both CPT and ERYC, choosing a concentration of 20 μM for both drugs. This is illustrated in Figure 2(B), where it is shown that the effect of ERYC occurs after 30 s and is maintained over time (lanes 9–16), whereas that of CPT occurs again after 30 s (lane 17), but it is nearly abolished after 8 min (lanes 22–23), indicating that the binding of ERYC and CPT is irreversible and reversible respectively. The relaxation assay in the presence of only DMSO shows that the solvent has no effect (Figure 2B, lanes 1–8).

The complete inhibition of the pre-incubated ERYC–enzyme also remains after a 3-fold or 9-fold dilution, confirming that the inhibitory effect is irreversible as shown in Figure 2(C, lanes 4–6), where it is also shown that the enzyme pre-incubated with DMSO, as a control, and diluted maintains its activity (lanes 1–3). As a control it was verified that the gel electrophoresis of DNA in the presence of ERYC (Figure 2C, lane 7) is identical with that of DNA alone (results not shown).

Effects of ERYC on cleavage/religation equilibrium

To investigate the effect of ERYC on the cleavage/religation equilibrium, the stability of the covalent DNA–enzyme complex was analysed using the 25 mer full duplex oligonucleotides substrate CL25 (5′-GAAAAAGACTTAGAGAAAAATTTT-3′)/CP25 in the presence of ERYC, CPT, or ERYC and CPT together (Figure 3). When the enzyme is incubated with DMSO (Figure 3, lane 1), a very small level of cleavage of the labelled DNA strand

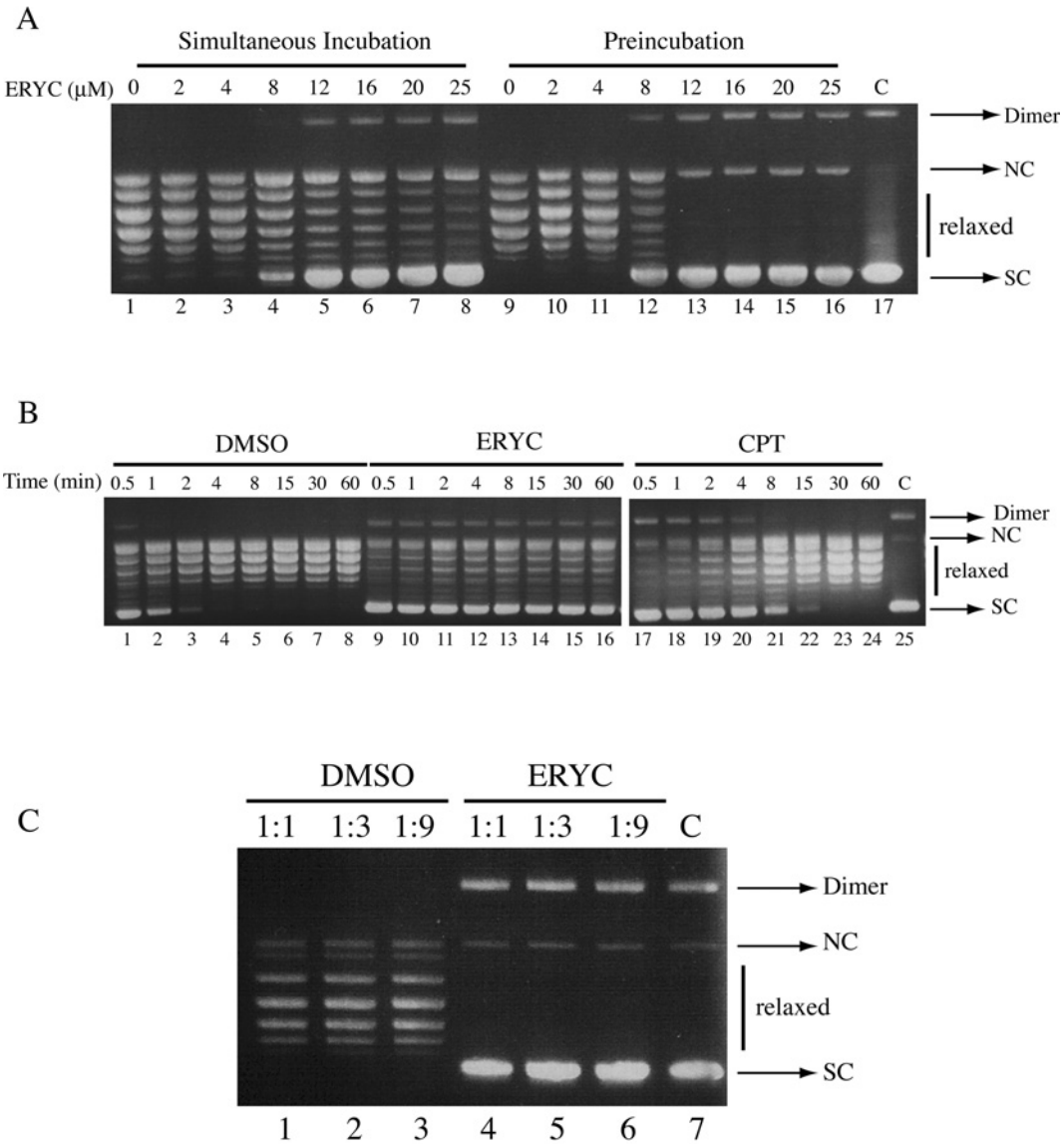

Figure 2 Relaxation of supercoiled DNA

(**A**) Relaxation of negative supercoiled plasmid DNA by Top1 with increasing concentrations of ERYC (lanes 2–8) or after pre-incubation for 10 min at 37 °C with increasing concentrations of ERYC (lanes 10–16). Lanes 1 and 9, no drug added; lane 17, no protein added. (**B**) Relaxation of negative supercoiled plasmid DNA in a time course experiment for DMSO (lanes 1–8), 20 μM ERYC (lanes 9–16) and 20 μM CPT (lanes 17–24); lane 25, no protein added. (**C**) Relaxation assay of Top1 preincubated with DMSO or ERYC (100 μM) for 10 min and then diluted 3- or 9-fold. Lane 7, no protein added. The reaction products are resolved in an agarose gel and visualized with ethidium bromide. Dimer, dimer supercoiled plasmid DNA; NC, nicked supercoiled plasmid DNA; SC, supercoiled plasmid DNA.

is detected at the preferred DNA cleavage site, as indicated by the asterisk in Figure 3. When Top1 is exposed to CPT, a dramatic increase in cleaved DNA fragments is observed (Figure 3, lane 2), indicating that the equilibrium is shifted towards cleavage, since the drug reversibly binds to the covalent intermediate DNA–enzyme, stabilizing the cleavable complex and reducing the religation rate [32]. When the protein is incubated with ERYC, the band of the cleavable complex is not observed at all (Figure 3, lane 3). Pre-incubation of ERYC with the enzyme before addition of substrate and CPT (Figure 3, lane 5) does not permit the stabilization of the cleavable complex by CPT, whereas pre-incubation of CPT with the enzyme before addition of substrate and ERYC (Figure 3, lane 4) does not alter the inhibition mechanism of CPT. The results can be interpreted as either that ERYC inhibits the cleavage or that it induces a faster religation, in both cases not permitting the cleavable complex stabilization.

Cleavage and religation assays in the absence and presence of ERYC

The cleavage of the enzyme as a function of ERYC concentrations was followed using a suicide cleavage substrate. In detail, a 5′-end radiolabelled oligonucleotide CL14 (5′-GAAAAA-AGAC*TT'AG-3′) was annealed to the CP25 (5′-TAAAAATTT-TTCTAAGTCTTTTTTC-3′) complementary strand, to produce a duplex with an 11-base 5′ single-strand extension. The enzyme cuts preferentially at the site indicated by the arrow and to a

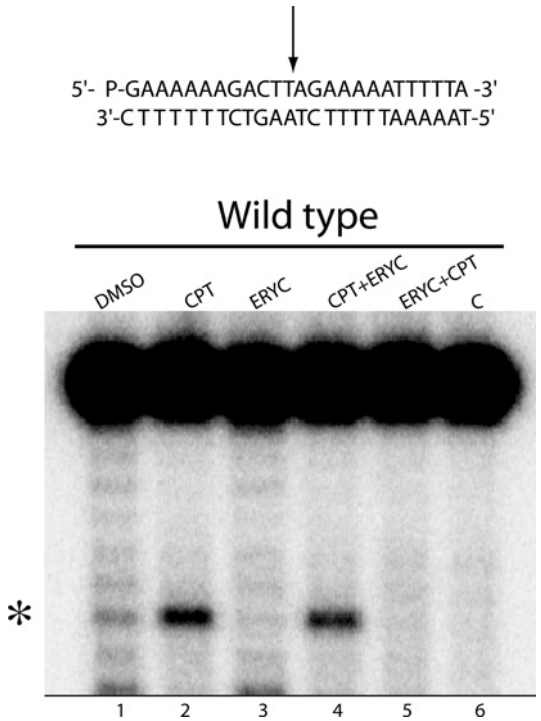

Figure 3 Cleavage/religation equlibrium

Gel electrophoresis of the products coming from the incubation of Top1 with the [γ-^{32}P] end-labelled duplex DNA, shown at the top of the Figure. The arrow on the DNA sequence indicates the CL1 preferred cleavage site. The duplex was incubated with the enzyme alone (lane 1), in the presence of CPT (lane 2), in the presence of ERYC (lane 3) after pre-incubation with CPT and then addition of ERYC (lane 4), and after pre-incubation with ERYC and then addition of CPT (lane 5). In the last lane the enzyme has not been added. The asterisk indicates the band corresponding to the CL1 site.

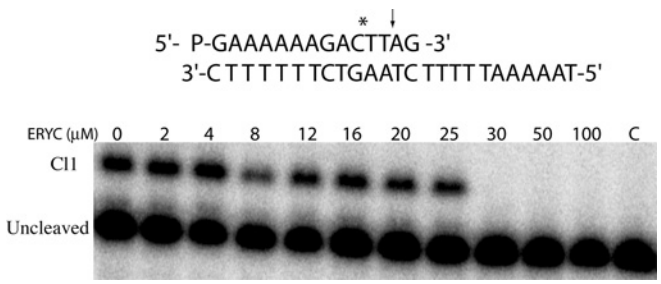

Figure 4 Suicide cleavage experiment in the presence of different concentrations of ERYC

An ERYC dose-dependent suicide cleavage reaction was carried out with the substrate described on the top of the Figure. Different concentrations of ERYC were pre-incubated with the protein for 10 min at 37 °C and then incubated with the substrate for 30 min. CL1 represents the DNA fragment cleaved at the preferred site. Lane C, no protein added. The arrow and the asterisk indicate the first and the second cleavage site respectively.

lower extent at the site indicated by the asterisk. In both cases, the religation step is precluded because the AG-3′ or TTAG-3′ oligonucleotides are too short to be religated, leaving the enzyme covalently attached to the 12- or 10-oligonucleotide 3′-end. The presence of the cleaved DNA fragments was monitored in the presence of different pre-incubated concentrations of ERYC in a denaturing polyacrylamide gel (Figure 4). The data indicate that increasing ERYC concentrations decreases the percentage of protein able to cleave the substrate, and that at 30 μM the enzyme is fully inhibited. This behaviour precludes the ability to carry out a study of the effect of ERYC on the cleavage rate, since at any concentrations lower than 30 μM there are two protein populations: a fully inhibited one having a strongly bound ERYC, and a fully unbound active protein.

The DNA religation step was studied by testing the ability of the wild-type protein to religate the R11 (5′-AGAAAAATTTT-3′) oligonucleotide added to the cleaved suicide substrate previously incubated with an excess of enzyme. The religation was followed in the absence of the drug or prior to incubation of the cleavable suicide complex with ERYC. Aliquots have been removed at different times, the reaction stopped by addition of SDS and the products analysed by PAGE (Figure 5). The Figure clearly shows that, in the absence of the drug, the wild-type protein religates the R11 oligonucleotide in a time-dependent manner, whereas the presence of the drug fully inhibits the religation step, confirming that also in this case ERYC irreversibly binds to the protein–DNA complex.

EMSA

The lack of any cleavage activity in the presence of ERYC could be due to an effect of the drug in preventing the binding of the protein to the DNA or to an inhibition of the catalytic reaction. In order to clarify this point, an EMSA was performed to explore whether ERYC prevents binding of Top1 to DNA. To this purpose, the inactive human recombinant mutant Y723F was pre-incubated with 100 μM ERYC for 10 min, a 25 bp double-stranded DNA has then been added and the samples have been resolved into a native polyacrylamide gel. In the absence of protein (Figure 6, lanes 1, 3, 5 and 7), no DNA shift was observed. In the presence of the Y723F mutant either in the absence (Figure 6, lane 2) or the presence of ERYC (Figure 6, lane 4), two slowly migrating Top1–DNA complexes are formed, corresponding to DNA bound to a full protein (Top1–DNA complex 1) and to DNA bound to a partially degraded form of the protein (Top1–DNA complex 2). The intensity of the band corresponding to the Top1–DNA complex 1 increases, doubling the protein concentrations (Figure 6, compare lanes 2 and 4 with lanes 6 and 8), being 17 for the Top1–DNA complex 1 in lanes 2 and 4 and 35 for the Top1–DNA complex1 in lanes 6 and 8, demonstrating that ERYC does not prevent the Top1–DNA complex formation.

Protein–ligand interaction

The interaction between Top1 and ERYC was explored by carrying out 250 runs of Molecular Docking on the structure of the protein in the absence of the DNA substrate. In more than 70 % of the results, ERYC is interacting with the same protein region (site I), near the loop connecting subdomain III with the linker domain (Figure 7A). In approx. 30 % of the complexes, another binding region is identified (site II) in the proximity of the segment connecting the cat and cap domains, formed by helices α8 and α9 (results not shown). The complexes belonging to the first binding site are characterized by a free energy value ranging from -4.6 to -6.6 kcal/mol (1 cal $\approx$ 4.184 J) that are on average 1 kcal/mol lower than that found in the second site, confirming site I as the preferential binding site.

Analysis of the contacts established between the protein and the ligand in the preferential binding site indicates that ERYC is in close contact with two residues of the catalytic pentad, Arg^{488} and His^{632}, through the drug prenilic substituent in position 8 (Figure 7B), providing a molecular explanation for the inhibitory effect of the drug. Indeed, these two residues are fundamental during the interaction with the scissile base, establishing contacts with the bridging and nonbridging oxygens

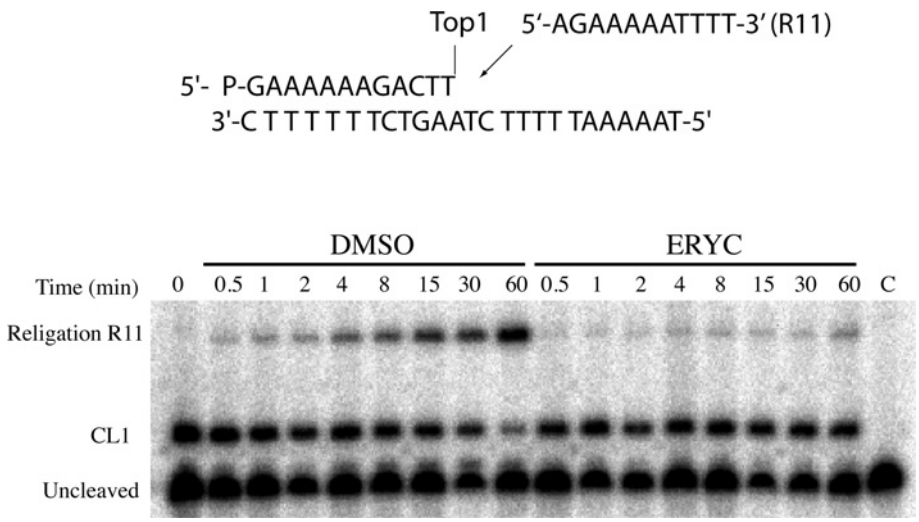

Figure 5 Religation experiment in the absence and presence of ERYC

Time course (0.5–60 min) of the religation experiment between the R11 substrate and the wild-type covalent complexes described at the top of the Figure, in the absence or presence of 100 μM ERYC. CL1 represents the DNA fragment cleaved at the preferred site. Religation represents the religated product.

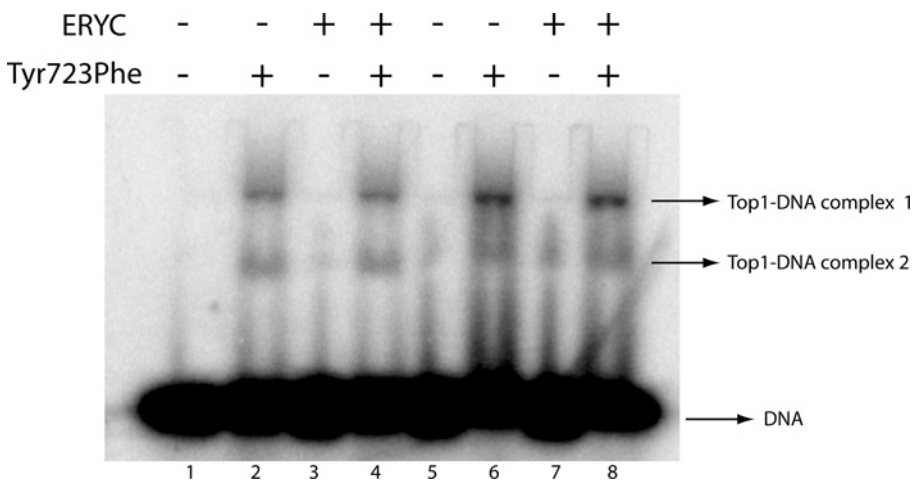

Figure 6 EMSA

EMSA of the [γ-^{32}P] end-labelled CL25/CP25 DNA substrate alone (lane 1); in presence of the inactive Y723F mutant (Tyr723Phe) (lane 2); of ERYC (lane 3); of both the drug and the mutant (lane 4). Lanes 6 and 8 represent the same experiment reported in lanes 2 and 4 but with twice the enzyme concentration than that in lanes 2 and 4. Top1–DNA complex 1 indicates the DNA bound to the complete Top1 protein, Top1–DNA complex 2 indicates the DNA bound to a degraded form of Top1 protein.

during the stabilization of the transition state [5,33,34]. Moreover, Arg^{488} was proposed to accept a proton from Tyr^{723}, either directly or via a water molecule, during the nucleophilic attack [34–36]. Binding of the drug in site I does not produce a steric hindrance, such as to impede the DNA binding. In fact, the DNA substrate can also be accommodated in the presence of the drug (results not shown), in agreement with the experimental findings and confirming that the inhibition effect is not due to prevention of the DNA binding, but to an effect on the enzyme catalytic site.

A docking experiment was also carried out between ERYC and the protein–DNA binary complex using the ternary complex X-ray structure [23] where the TPT drug was eliminated. A total of 250 docking runs were carried out for ERYC, TPT or CPT. In the case of ERYC and TPT, two main binding sites were identified (Figures 8A and 8B), one with the drug intercalating between the base pairs in position $-1/+1$, in a position similar to the one found in the X-ray structure of the ternary complex, the other one in the same site identified for the free enzyme (site I) (Figures 7, 8A and 8B). The binding energy for ERYC in these two sites is comparable and of the order of -7 kcal/mol. The binding energy for the two sites is comparable for TPT as well, but it is less favourable than ERYC by approx. 3 kcal/mol, suggesting that ERYC can be a suitable scaffold to be used as a Top1 poison. Interestingly, in the case of CPT in all of the 250 docked structures, the drug intercalates between the $+1/-1$ bases, and the other site is not visible at all (Figure 8C), suggesting that CPT is highly specific as confirmed by the binding energy that varies between -7.7 and -10.7 kcal/mol, for all the 250 complexes.

DISCUSSION

The results shown in the present study indicate that ERYC inhibits both cleavage and religation reactions catalysed by human

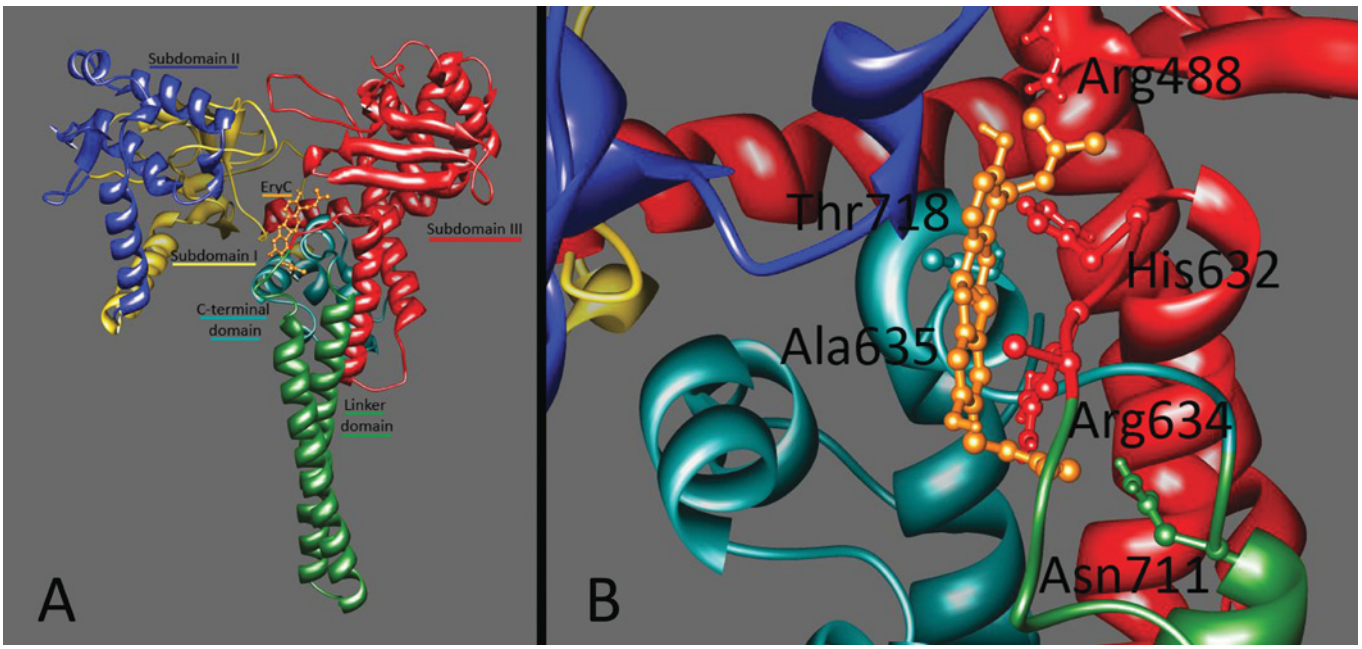

Figure 7 Top1–ERYC binding pocket

(**A**) Ribbon representation of the protein with subdomains I, II and III coloured in yellow, blue and red respectively and the linker and C-terminal domain coloured in green and cyan respectively. The best docked ERYC–Top1 complex is represented in orange in ball and stick. (**B**) A close up of the binding pocket: the lateral chains of the residues contacting the drug are represented as ball and stick and coloured following the domain colour code.

topoisomerase IB. The drug is then able to interact with the enzyme alone or with the DNA–enzyme-cleavable complex, in both cases inhibiting the enzyme active site. The drug is acting with a mechanism completely different to the drugs belonging to the CPT family drugs, which are probably better characterized for this class of enzymes [37]. CPT is classified as a poison, since it converts topoisomerase into a cell poison, reversibly binding at the interface of the DNA–topoisomerase cleavable complex, trapping it long enough to permit collision with the replication fork [38]. The mechanism of CPT can be easily understood observing Figure 3, where the band of the cleavable complex, stabilized by the drug, appears (Figure 3, lane 2). On the other hand, such a band is not observed in the presence of ERYC (Figure 3, lane 3) or even after addition of CPT to an enzyme pre-incubated with ERYC, suggesting that ERYC does not permit the cleavage of DNA because of a direct interaction between the drug and the enzyme. This is one of the differences between ERYC and CPT, ERYC is able to interact with the enzyme alone, binding in proximity of the active site and inhibiting the cleavage process, whereas CPT does not bind to the free enzyme and only binds to the DNA–enzyme-cleavable complex. Another interesting difference concerns the reversibility: CPT binds in a reversible way, whereas binding of ERYC is irreversible. The irreversibility of the ERYC binding can be observed either monitoring the relaxation (Figure 2), the cleavage (Figure 4) or the religation (Figure 5) reactions. Complete inhibition of the relaxation process can in fact be achieved only by pre-incubating the enzyme with ERYC (Figure 2A). Moreover, the inhibition also remains after a 9-fold dilution, at a concentration where the enzyme, in the absence of the drug, is active (Figure 2C), confirming that the ERYC binding is irreversible. The same indication is obtained when the drug is incubated with the cleavable suicide complex (Figure 5): in the absence of the drug, the enzyme religates in a time-dependent manner, whereas in the presence of the drug, the religation step is constantly inhibited at any time. It is likely that the binding of the drug induces some enzyme conformational change that renders the binding irreversible.

The docking of the ERYC molecule to Top1 provides a molecular explanation for the inhibition effect although cannot provide clear information for the irreversibility. For the enzyme alone the preferential drug binding site is found near the two active site residues Arg^{488} and His^{632} (Figure 7), both implicated in the reaction catalysis, possibly inhibiting their action. It is interesting to notice that once the molecule is bound to the enzyme, there is still space to accommodate the DNA substrate into the cavity (results not shown) in perfect agreement with the experimental results that indicate that the presence of ERYC does not prevent the DNA binding (Figure 6).

Docking of the drug with the DNA–enzyme cleavable complex indicates the occurrence of two binding sites, one intercalating the $-1/+1$ cleaved base pair, the other one identical with the binding site found in the free enzyme (Figures 7A and 8A). The docking procedure can then explain the inhibitory property of the drug, but not its irreversibility. However, it is also interesting to remark that the binding energy indicates that ERYC binds the cleavable complex even more efficiently than TPT suggesting that it can be a potential scaffold to be used for therapeutic purpose.

The results from the present study indicate that ERYC is *in vitro* a catalytic inhibitor of human Top1, efficiently acting both on the cleavage and on the religation reaction. However, these results do not indicate how much this inhibition contributes to the cytotoxicity of the drug itself. In fact the primary mechanism of cell killing by Top1 poison, as CPT, is S-phase-specific [38] through potentially lethal collisions between advancing replication forks and Top1-cleavable complexes. ERYC was found to induce a dose-dependent cytotoxicity unrelated to

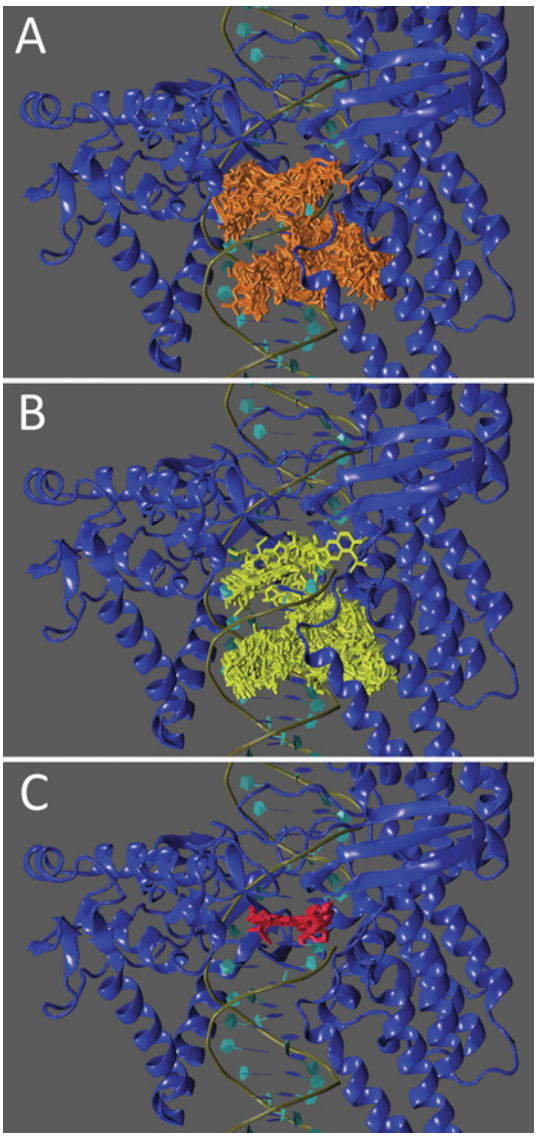

Figure 8 Docking of Top1–DNA binary complex with ERYC, TPT or CPT

Spread of the docked structure of ERYC (**A**), TPT (**B**) or CPT **(C)** in the binary complex coming from the 1k4t structure. The protein and DNA are represented as blue and cyan ribbon respectively. ERYC (**A**), TPT (**B**) and CPT (**C**) are represented in orange, yellow and red respectively.

genotype status of the cell lines and without apparent cell cycle check-point arrest [9]. It is then likely that, in cells, Top1 is not the only target of ERYC and that the observed apoptosis is due to the interaction of multiple molecular targets.

In conclusion, we have characterized the *in vitro* interaction of ERYC with Top1, demonstrating that this drug has unique properties, being able to act either as a cleavage or religation inhibitor, suggesting that appropriate chemical modifications can be introduced to confer the ability to act on a single catalytic step process to the drug.

AUTHOR CONTRIBUTION

All experiments where performed by Cinzia Tesauro under the supervision of Paola Fiorani and Alessandro Desideri, the Molecular Docking experiments where performed by Ilda D'Annessa under the supervision of Giovanni Chillemi. Figures were prepared by Paola Fiorani and Ilda D'annessa. ERYC was kindly provided by Gino Turchi.

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online data

SUPPLEMENTARY ONLINE DATA

Erybraedin C, a natural compound from the plant *Bituminaria bituminosa*, inhibits both the cleavage and religation activities of human topoisomerase I

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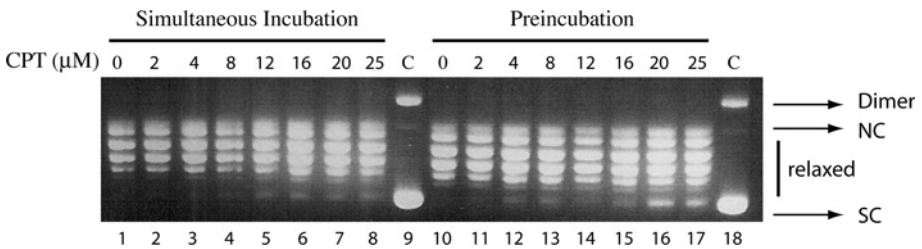

Figure S1 Relaxation of negative supercoiled plasmid DNA by Top1

The effect of increasing concentrations of CPT (lanes 2–8) or after pre-incubation for 10 min at 37°C with increasing concentration of CPT (lanes 10–17). Lanes 1 and 10, no drug added; lanes 9 and 18, no protein added.

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