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A Natural Anticancer Agent Thaspine Targets Human Topoisomerase IB

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Abstract: The different steps of the topoisomerase I catalytic cycle have been analyzed in the presence of the plant alkaloid thaspine (1-(2-(Dimethylamino)ethyl)-3,8-dimethoxychromeno[5,4,3-cde]chromene-5,10-dione), known to induce apoptosis in colon carcinoma cells. The experiments indicate that thaspine inhibits both the cleavage and the religation steps of the enzyme reaction. The inhibition is reversible and the effect is enhanced upon pre-incubation. Molecular docking simulations of thaspine over topoisomerase I, in the presence or absence of the DNA substrate, show that thaspine, when interacting with the enzyme alone in the closed or in the open state, can bind in proximity of the active residues preventing the cleavage reaction, whilst when docked with the enzyme-DNA cleavable complex intercalates between the DNA bases in a way similar to that found for camptothecin, explaining its religation inhibition. These results unequivocally demonstrate that thaspine targets human topoisomerase I .

Keywords: Anticancer drug, Camptothecin, Human topoisomerase IB, Inhibitor, Molecular docking, Thaspine.

INTRODUCTION

Topoisomerases are a family of enzymes that control the topological state of DNA during crucial cellular processes such as replication, transcription, recombination and chromosomal segregation [1,2]. Topoisomerases are divided in two subfamilies depending on their mechanism of action, type II topoisomerases cleave and reseal two DNA strands, while type I topoisomerases cleave only a single strand of the double DNA helix. Within each subfamilies two different classes can be identified: 1) type A enzymes form a covalent bond with the 5' end of the DNA generated at the cleavage site, 2) type B form a covalent bond with the 3' end.

Human topoisomerase IB is an essential enzyme involved in the modulation of the topological state of DNA, modulation of gene transcription, phosphorylation of the SF2/ASF_splicing factor and DNA repair [3-5]. The human enzyme is composed of 765 amino acids organized in four different domains: N-terminal domain (1-214), linker domain (636-712) C-terminal domain (713-765) and core domain (215-635) [1], the last one being further divided in three sub-domains (I, II and III) [1,6]. The enzyme has a final bilobed structure, one lobe is composed by subdomain I and II and it is called "cap", the other one is composed by sub-domain III and C-terminal domain and it is called "cat" [7].

Topoisomerase IB is of great clinical interest, since it is the only cellular target of camptothecin (CPT), a natural compound extracted from bark and fruits of an Asian plant, *Camptotheca acuminate*. Two water soluble CPT derivatives, topotecan and irinotecan, have been approved by the Food and Drug Administration and are in clinical use for the treatment of various solid tumors. Camptothecin acts through the stabilization of the covalent enzyme-DNA binary complex, inhibiting the resealing of the cleaved strand and transforming the enzyme into a cell poison [8-10]. During the S-phase of the cell cycle, collision of the replication fork with the drug-stabilized complexes converts a transient single strand break into an irreversible double helix break, resulting in cell death. These kinds of drugs are commonly referred to as "poisoners". Other compounds can inhibit topoisomerase IB by different mechanisms, directly interacting with the enzyme and/or the DNA, preventing other catalytic cycle steps of the topoisomerase IB, such as the cleavage reaction. These compounds are usually classified as "catalytic inhibitors".

Several natural and non-natural compounds, such as terpenoids, flavonoids, stilbenes, fatty acids, and transition metal complex have been shown to interact with topoisomerase IB and act as poisoners and/or catalytic inhibitors [11-15]. Natural products are particularly interesting since they have been selected during evolution to interact with biological targets. Furthermore their high degree of chemical diversity makes them attractive as lead compounds for development of new drugs. Recently upon screening a library of natural compounds, thaspine (also referred to as taspine) (Fig. **1**), an alkaloid present in the cortex of the South American tree *Croton lechleri* and used in traditional medicine for different purposes, was shown to induce apoptosis in HCT116 colon carcinoma cells [16]. In the same study thaspine was shown to induce a gene expression profile similar to that induced by CPT, suggesting a similar cytotoxic mechanism.

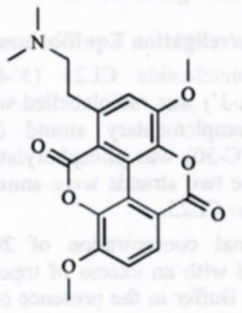

Fig. (1). Chemical structure of thaspine.

In this study we have analyzed the interaction between thaspine and purified topoisomerase I, investigating the different steps of the enzyme catalytic cycle, showing that thaspine inhibits both the cleavage and the religation processes. Molecular docking simulation indicates that thaspine, when interacting with the enzyme alone, can bind in proximity of the active site preventing

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the cleavage reaction, whilst with the enzyme-DNA cleavable complex intercalates between the DNA basis in a way similar to CPT explaining its religation inhibition and unambiguously demonstrating that thaspine is targeting human topoisomerase IB.

MATERIALS AND METHODS

Thaspine (NSC76022) was obtained from the Developmental Therapeutics Program at the National Cancer Institute (http://www.dtp.nci.nih.gov) and dissolved in DMSO to a final concentration of 10 mM and stored at −20°C.

Expression and Purification of Human Topoisomerase IB

Human topoisomerase IB was expressed under a GAL-1 inducible promoter by a single-copy plasmid in EKY3 transformed cells, as previously described [17].

The protein was purified using an ANTI-FLAG M2 Affinity Gel column (Sigma), since it contains an N-terminal sequence DYKDDDY recognized by the M2 monoclonal antibodies [14].

DNA Relaxation Assays

Human topoisomerase I activity was analyzed incubating the enzyme with 0.5 μg of negatively supercoiled pBlue-Script KSII(+) in 30 μl of 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).

The effect of thaspine on topoisomerase activity was measured by adding different concentrations of the compound, at different times. The reactions were stopped with 0.5% SDS after 30 min or after each time-course point 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 pre-incubated at 37 °C for 5 min, prior to the addition of the substrate, the mixture was then incubated at 37 °C for 30 min. The reversibility of thaspine-topoisomerase I interaction was monitored after pre-incubation for 5 min at 37 °C, the inhibitor-topoisomerase mixture was then diluted in the reaction buffer, the DNA substrate was added and incubated at 37 °C for 30 min. Where indicated the samples were stopped by addition of 1M NaCl and incubated further at room temperature for 5 min. When necessary they were run in the presence of ethidium bromide [18].

Assays were performed at least three times but only one representative gel is shown.

Cleavage/religation Equilibrium Assay

Oligonucleotide CL25 (5'-GAAAAAAGACTTAGAAAAA-TTTTTA-3') was radiolabelled with [γ-^{32}P]ATP at its 5' end. The CP25 complementary strand (5'-TAAAAATTTTTCTAAGTC-TTTTTTC-30) was phosphorylated at its 50 end with unlabeled 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 topoisomerase I enzyme at 37 °C in Reaction Buffer in the presence or absence of 50 μM thaspine and 50 μM CPT. Dimethyl sulfoxide (DMSO) was added to no drug controls. At different time the reactions were stopped with 0.5% SDS and digested with trypsin after ethanol precipitation. Reaction products were resolved in 20% acrylamide-7 M urea gel. The experiments have been repeated at least three times.

Kinetics of Cleavage

The oligonucleotide CL14 (5'-GAAAAAAGACTTAG-3') was radiolabelled with [γ-^{32}P]ATP at its 5' end. The CP25 complementary strand 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 substrate called "suicide substrate" contains a partial duplex.

The suicide cleavage reactions were carried out incubating 20 nM DNA with an excess of enzyme in Reaction Buffer at 37 °C and in presence of 50 μM thaspine. DMSO was added to no-drug control. Where indicated thaspine was pre-incubated with the enzyme for 5 minutes before DNA addition. At different time points 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 6 μl of 1 mg/ml trypsin and incubated at 37 °C for 1 h. Samples were analyzed using 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 for each time point, comparing the intensity of the CL1 band, normalized on the total amount of radioactivity in each lane, with the maximum CL1 value obtained in the presence of only DMSO at the longest times.

Kinetics of Religation

The suicide CL14/CP25 substrate (20 nM), prepared as above, was incubated with an excess of topoisomerase IB enzyme for 30 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 50 μM thaspine, permitting to the enzyme to perform the religation step restoring a full duplex oligonucleotide as the final product. DMSO was added to no-drug controls. At time-course 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 1 h. 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 to the total amount of radioactivity in each lane and relative to the highest amount of substrate converted to reaction product by human topoisomerase IB in the presence of only DMSO.

Molecular Docking Procedure

Protein-ligand molecular docking, a computational technique that predicts the putative binding site of a ligand on a receptor taking into account both geometrical and electrostatic match contributions, has been used to predict the complexes between the human topoisomerase I enzyme and the thaspine drug (1-[2-(dimethylamino)ethyl]-3,8-dimethoxychromeno[5,4,3-cde]chromene-5,10-dione). The protein-ligand docking has been conducted with the AutoDock 4 program [19], using the AutoDockTools suite version 4 [20]. The docking runs have been carried out using the crystal structure of the human topoisomerase I (PDB ID: 1K4T) [21], removing the topotecan drug and in presence or absence of the 22 base pair DNA duplex. In addition, the last frame of the open state structure of human topoisomerase I, obtained by molecular dynamics (MD) simulation [22] of the PDB structure 1A36 [23], after the removal of the 22 base pair DNA duplex, has been used to evaluate the interaction energy of the thaspine drug with the enzyme in its open structure before its interaction with the DNA substrate. Four docking simulations have been carried out: docking I, thaspine versus 1K4T without topotecan but with DNA duplex; docking II, thaspine versus 1K4T without topotecan and without DNA duplex; docking III, thaspine versus 1A36, open state from MD simulation, in the active site zone; docking IV, thaspine versus 1A36, open state from MD simulation, in the nose cone zone. 250 docking runs have been performed for each simulation using the Lamarckian Genetic

Algorithm and a grid point spacing of 0.375 Å. For docking number I and II a box of 45 x 45 x 45 (grid points in x, y, z) has been centred in the nicked DNA cavity obtained after the removal of the topotecan drug or of the topotecan and the DNA substrate; for docking number III and IV boxes of 95 x 45 x 95 and 68 x 126 x 126 (grid points in x, y, z), have been centred in the active site and in the nose cone region, respectively.

RESULTS

Inhibitory Effect of Thaspine on Topoisomerase IB Activity

The inhibitory effect of thaspine on topoisomerase IB activity, analyzed through a supercoiled plasmid relaxation assay where wild-type enzyme is incubated with different concentrations of thaspine, is shown in Fig. (**2A**). The assay shows that thaspine inhibits topoisomerase I activity in a dose-dependent manner. Weak inhibition of enzyme activity is observed at a concentration of 10 μM and higher concentrations lead to complete inhibition. Since thaspine is dissolved in DMSO, control experiments were performed to ensure that an equal concentration of the solvent did not affect the relaxation activity of topoisomerase I (Fig. **2A**, lane 2). Thaspine can fully inhibit topoisomerase I at concentrations lower than 15 μM if it is pre-incubated with the enzyme (Fig. **2B**), as shown by a comparative time course relaxation experiment carried out with and without enzyme–compound pre-incubation. In fact, 10 μM thaspine fully inhibits the enzyme when pre-incubated with the enzyme for 5 minutes before substrate addition (Fig. **2B**, lanes 10-13), whilst no effect is observed in absence of pre-incubation (Fig. **2B**, lanes 6-9). This result indicates that thaspine is able to directly interact with topoisomerase. This is in contrast to camptothecin, which selectively interacts with the covalent DNA-enzyme binary complex [24].

In order to verify that the topoisomerase I -thaspine interaction is reversible, the pre-incubated thaspine-topoisomerase I solution was serially two-fold diluted, prior to adding the substrate (Fig. **2C**). The result shows that incubation of 50 μM thaspine completely inhibits the activity of the enzyme (Fig. **2C**, lane 6), but the effect is lost after dilution, indicating that the enzyme-

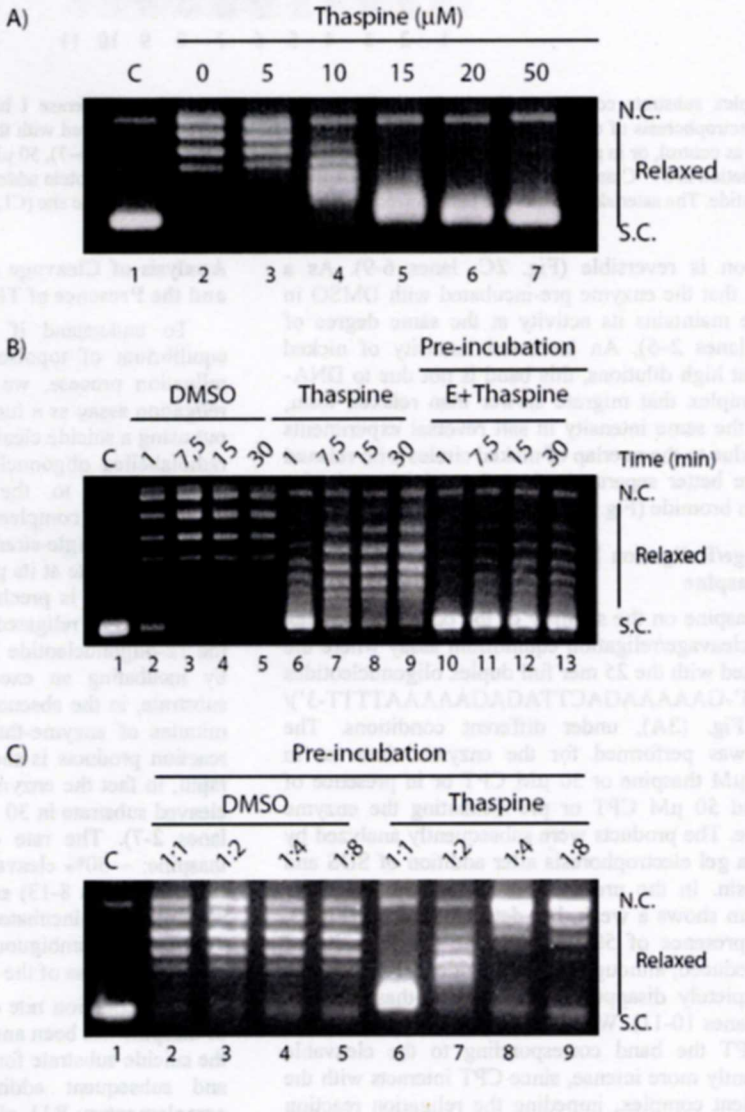

Fig. (2). **(A)** Relaxation of negative supercoiled plasmid DNA by topoisomerase I in the presence of increasing concentrations of thaspine (lanes 3–7). Lane 1, only substrate. Lane 2, control reaction with DNA enzyme and DMSO in the absence of thaspine. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA. **(B)** Relaxation activity assay of topoisomerase IB on negative supercoiled plasmid in presence of only DMSO (lanes 2–5), 10 μM thaspine (lanes 6–9), after 5 min enzyme pre-incubation with 10 μM thaspine (lanes 10–13). Lane 1, only substrate. **(C)** Relaxation assay of topoisomerase IB pre-incubated with only DMSO or thaspine (50 μM) for 1 min and then serially diluted up to 8-fold. Lanes 1, only substrate. NC, nicked circular plasmid DNA. SC, supercoiled plasmid DNA.

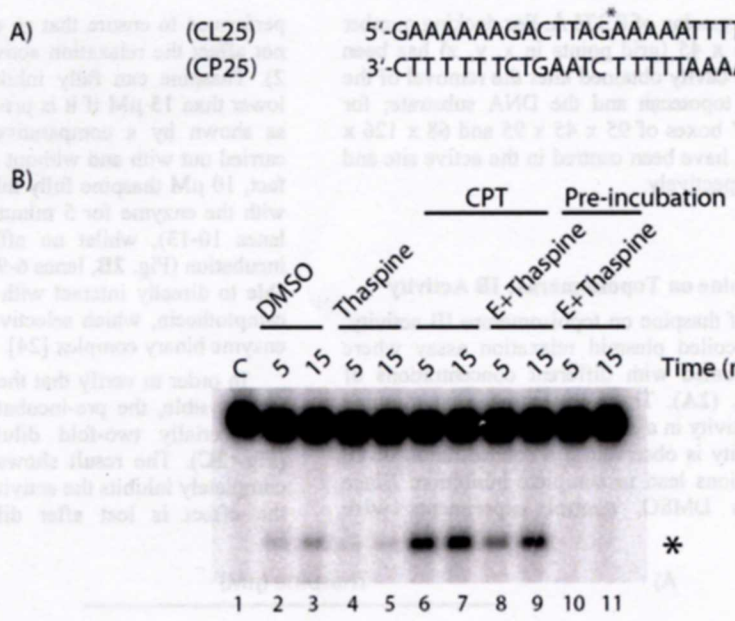

Fig. (3). **A)** Full duplex substrate containing 5'end-labeled CL25 and the preferred topoisomerase I binding site, indicated by an asterisk. **B)** Urea–polyacrylamide gel electrophoresis of cleavage/religation equilibrium reaction. Substrate is incubated with the enzyme at 37 °C for 5, 15 minutes in presence of DMSO (lanes 2–3) as control, or in presence of 50 μM thaspine (lanes 4–5), 50 μM CPT (lanes 6–7), 50 μM thaspine and 50 μM CPT (lanes 8-9), or after 5 min enzyme pre-incubation at 37 °C and subsequent addition of DNA (lanes 10–11). Lane 1, no protein added. The slowest migrating band corresponds to the uncleaved oligonucleotide. The asterisk indicates the band corresponding to the preferential cleavage site (CL).

compound interaction is reversible (Fig. **2C**, lanes 6-9). As a control, it is shown that the enzyme pre-incubated with DMSO in absence of thaspine maintains its activity at the same degree of dilution (Fig. **2C**, lanes 2–5). An increased intensity of nicked circles is observed at high dilutions, this band is not due to DNA-topoisomerase I complex that migrate slower than relaxed form, since it remains of the same intensity in salt reversal experiments (Fig. **S1A**), but it is due to the overlap of nicked circles with relaxed topoisomers that are better separated when the gel is run in the presence of ethidium bromide (Fig. **S1B**) [18].

Analysis of Cleavage/Religation Equilibrium in the Absence or the Presence of Thaspine

The effect of thaspine on the stability of the covalent complex was analyzed by a cleavage/religation equilibrium assay where the enzyme was incubated with the 25 mer full duplex oligonucleotides substrate CL25 (5'-GAAAAAGACTTAGAGAAAAATTTT-3')/ CP25, shown in Fig. (**3A**), under different conditions. The equilibrium assay was performed for the enzyme alone or in the presence of 50 μM thaspine or 50 μM CPT or in presence of 50 μM thaspine and 50 μM CPT or pre-incubating the enzyme with 50 μM thaspine. The products were subsequently analyzed by polyacrylamide–urea gel electrophoresis after addition of SDS and digestion with trypsin. In the presence of DMSO the cleavage/ religation equilibrium shows a weak, but detectable, band (Fig. **3**, lanes 2-3). In the presence of 50 μM thaspine the intensity of the band is further reduced, although it is still evident (Fig. **3**, lanes 4-5), whilst it completely disappears after enzyme-thaspine pre-incubation (Fig. **3**, lanes 10-11). When the reaction is performed in the presence of CPT the band corresponding to the cleavable complex is significantly more intense, since CPT interacts with the DNA-enzyme covalent complex, impeding the religation reaction (Fig. **3**, lanes 6-7). In the presence of both thaspine and CPT the band corresponding to the cleaved complex is reduced when compared to CPT alone indicating that thaspine is not able to fully inhibit cleavage so that a part of the cleaved complex can be stabilized by CPT.

Analysis of Cleavage and Religation Reactions in the Absence and the Presence of Thaspine

To understand if thaspine modifies the cleavage/religation equilibrium of topoisomerase I perturbing the cleavage or the religation process, we have carried out either a cleavage or a religation assay as a function of time. The cleavage assay is carried out using a suicide cleavage substrate (Fig. **4A**), containing a 5'-end radiolabelled oligonucleotide CL14 (5'-GAAAAAAGACTT*AG-3') annealed to the CP25 (5'-TAAAAATTTTTCTAAGTC-TTTTTTC-3') complementary strand, to produce a duplex with an 11-base 5' single-strand extension. When the enzyme cuts the suicide substrate at its preferential site, indicated by an asterisk, the religation step is precluded because the AG-3' oligonucleotide is too short to be religated, leaving the enzyme covalently attached to the 12-oligonucleotide 3'-end. The cleavage kinetics was analyzed by incubating an excess of topoisomerase I with the suicide substrate, in the absence or presence of 50 μM thaspine or after 5 minutes of enzyme-thaspine pre-incubation. Gel analysis of the reaction products is shown in Fig. (**4B**). The cleavage reaction is rapid, in fact the enzyme cuts ~ 75% of the maximum quantity of cleaved substrate in 30 seconds in the absence of thaspine (Fig. **4B**, lanes 2-7). The rate of the reaction decreases in presence of thaspine: ~ 50% cleavage is observed in the presence of thaspine (Fig. **4B**, lanes 8-13) and is reduced to ~ 13% cleavage when the enzyme is pre-incubated with the inhibitor (Fig. **4B**, lanes 14-19). This assay unambiguously demonstrates that thaspine inhibits the cleavage process of the enzyme.

The religation rate of topoisomerase I in absence and presence of thaspine has been analyzed, incubating an excess of enzyme with the suicide substrate for 30 minutes to produce the suicide complex and subsequent addition of 200-fold molar excess of the complementary R11 oligonucleotide (5'-AGAAAAATTTT-3'), in presence or absence of 50 μM thaspine (Fig. **5A**) and further incubation for different times. Urea–polyacrylamide gel electrophoresis of the reaction products indicates that religation rate is slower in the presence of thaspine (Fig. **5B**, lanes 9-15) compared to the control (Fig. **5B**, lanes 2-8), showing that the compound

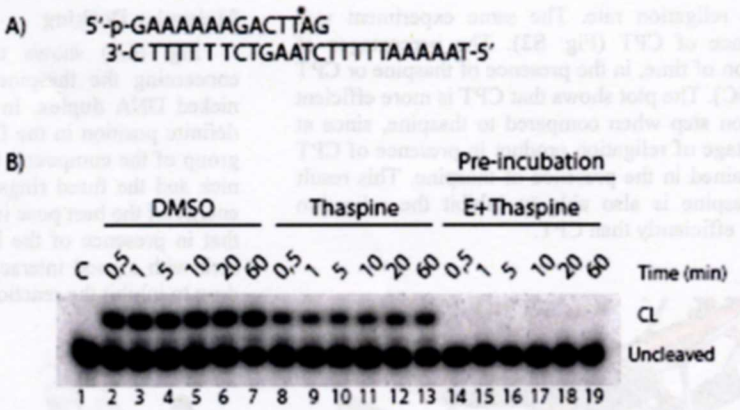

Fig. (4). A) The CL14/CP25 suicide substrate used to measure the cleavage kinetics of the enzyme, the preferred topoisomerase I binding site is indicated by an asterisk. **B)** Suicide cleavage kinetics with the CL14/CP25 substrate for topoisomerase I alone (lanes 2–7), in presence of 50 μM thaspine (lanes 8–13), or after 5 min enzyme-thaspine pre-incubation (lanes 14–19). Lane 1, substrate. CL represents the DNA fragment cleaved at the preferred enzyme site.

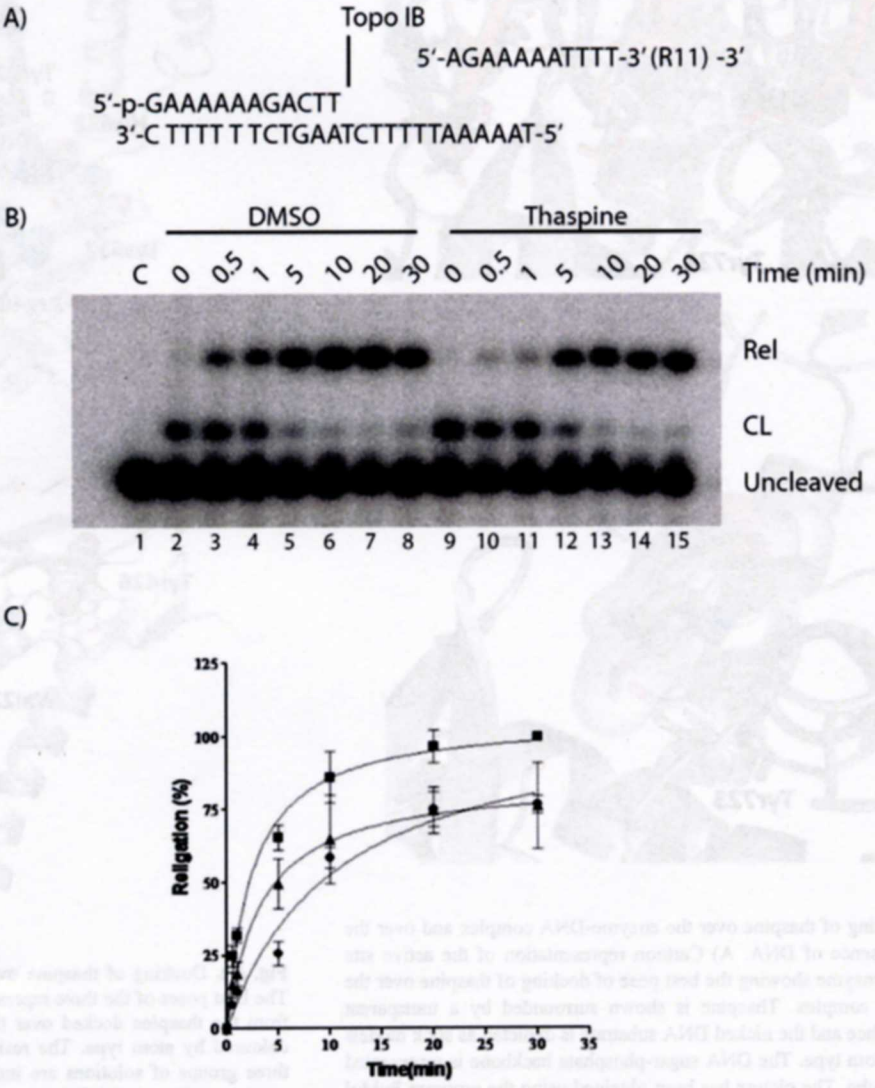

Fig. (5). A) The CL14/CP25 suicide substrate and the R11 complementary oligonucleotide used to measure the religation kinetics of the enzyme. **B)** Gel analysis of the religation kinetics for topoisomerase I in absence (lanes 2–8) or presence of 50 μM thaspine (lanes 9–15). CL represents the DNA fragment cleaved at the preferred enzyme site. Rel represents the religation product. **C)** Percentage of the religation product plotted at different times for topoisomerase IB in the absence of drugs (squares), in the presence of 50 μM thaspine (triangles), or in the presence of 50 μM CPT (circles)(The gel corresponding to the experiment in the presence of CPT is presented in Fig. **S2**). The data reported are the average ± SD of three independent experiments.

partially inhibits the religation rate. The same experiment was carried out in presence of CPT (Fig. **S2**). The percentages of religation, as a function of time, in the presence of thaspine or CPT are reported in Fig. (**5C**). The plot shows that CPT is more efficient to inhibit the religation step when compared to thaspine, since at short time the percentage of religation product in presence of CPT is about half that obtained in the presence of thaspine. This result demonstrates that thaspine is also able to inhibit the religation process, although less efficiently than CPT.

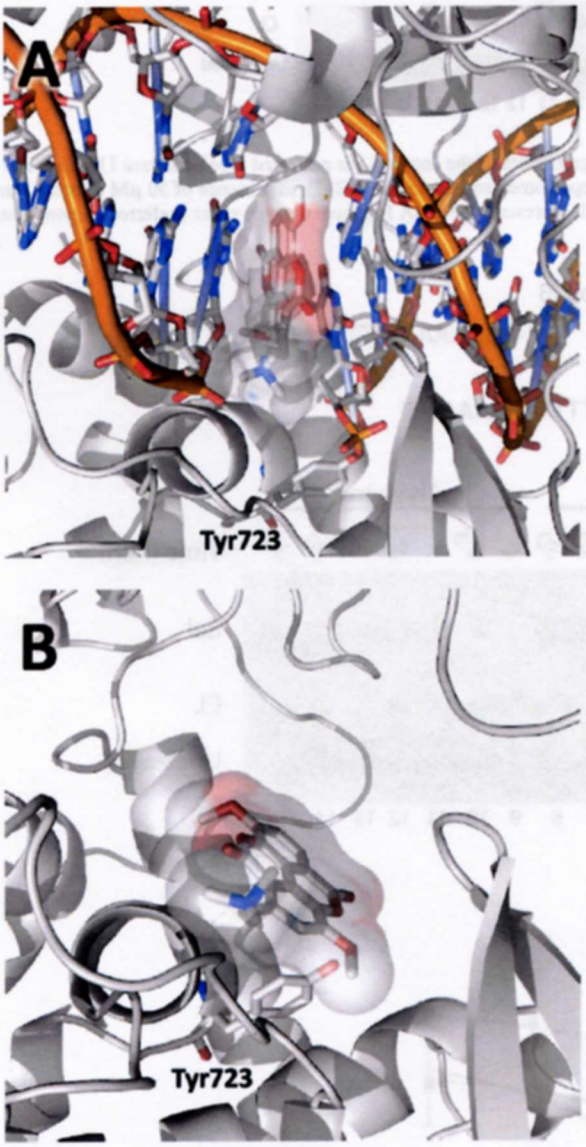

Fig. (6). Docking of thaspine over the enzyme-DNA complex and over the enzyme in absence of DNA. A) Cartoon representation of the active site region of the enzyme showing the best pose of docking of thaspine over the enzyme-DNA complex. Thaspine is shown surrounded by a transparent molecular surface and the nicked DNA substrate is depicted as stick models coloured by atom type. The DNA sugar-phosphate backbone is represented as an orange tube. The picture has been obtained using the program PyMol [25]. B) Cartoon representation of the active site region of the enzyme showing the best pose of the thaspine docked in absence of DNA. The thaspine drug is shown surrounded by a transparent molecular surface and the catalytic Tyr723 is depicted as a stick model coloured by atom type. The picture has been obtained using the program PyMol [25].

Molecular Docking

Fig. (**6A**) shows the best pose of all the docking runs concerning the thaspine versus the enzyme in presence of the nicked DNA duplex. In all the docking runs thaspine achieves a definite position in the DNA nick, with the 2-dimethylamino-ethyl group of the compound located between the two phosphates of the nick and the fused rings structure stacked between the bases. The energy of the best pose is about -11 kcal/mol. These results indicate that in presence of the DNA duplex, thaspine docks in the DNA nick with a good interaction energy that explains the ability of the drug to inhibit the reaction of religation (Fig. **5**).

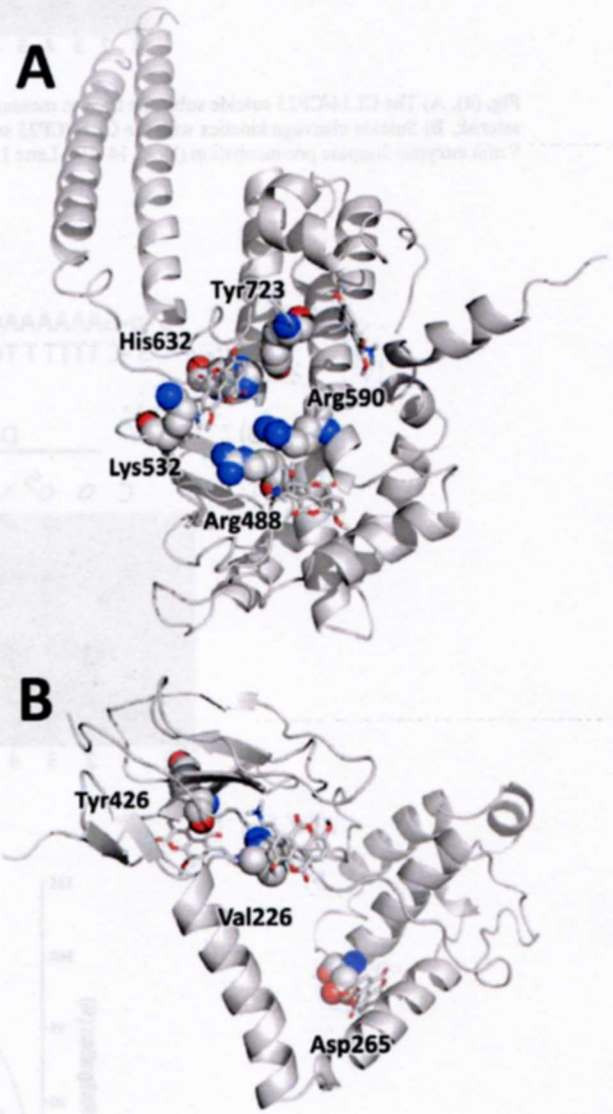

Fig. (7). Docking of thaspine over the enzyme "cat" and "cap" lobes. A) The best poses of the three representatives groups of the solutions, obtained from the thaspine docked over the "cat" lobe, are shown as stick models coloured by atom type. The residues of the catalytic pentad, close to the three groups of solutions are indicated as a spacefill models coloured by atom type. The picture has been obtained using the program PyMol [25]. B) The best poses of the three representatives groups, obtained from the thaspine docked over the "cap" lobe, are shown as stick models coloured by atom type. The residues close to each of the three groups of solutions are indicated as spacefill models coloured by atom type. The picture has been obtained using the program PyMol [25].

Since thaspine inhibits the cleavage reaction as well, the drug was also docked in absence of DNA with the enzyme in the closed state or in the open form, as obtained by a previous MD simulation [22]. Fig. (**6B**) shows the best pose of the docking between thaspine and the enzyme in its closed form without DNA duplex. In all the docking runs thaspine is flattened over the active site surface, in the proximity of Tyr723 and the best pose has an energy of about -5 kcal/mol.

In the case of the open state the drug has been separately docked over the two globular domains represented by the "cat " and the "cap" lobes. Fig. (**7A**) shows the docking between thaspine and the "cat" lobe of the enzyme and shows the three best drug poses, representing three groups of solutions, that spread over the active site close to the catalytic pentad residues. The first group of solutions is in proximity of the catalytic Tyr723 and its best pose is shown in the right side of Fig. (**7A**); the second group is located between Lys532 and His632, two residues belonging to the catalytic pentad, and its best pose is shown in the left side of Fig. (**7A**); the third group is located in proximity of Arg488 and Arg590, other two residues forming the catalytic pentad, and its best pose is shown in the bottom side of Fig. (**7A**). The interaction energies of these three solutions range between -6 and -7 kcal/mol.

Fig. (**7B**) shows the docking between thaspine and the "cap" lobe of the enzyme and shows the three best poses of the drug, representing three groups of solutions, lying over the nose cone helices. The first group of solutions is in proximity of Val226 and its best pose is shown in the centre of Fig. (**7B**); the second group is in proximity of Asp265 and its best pose is shown in the bottom side of Fig. (**7B**); the third group is in proximity of Tyr426 and its best pose is shown in the left side of Fig. (**7B**). The interaction energies of these three solutions is about -7 kcal/mol. These results indicate that the drug can bind with a good interaction energy with the open form of the enzyme before its interaction with the DNA substrate.

DISCUSSION

We here show that the plant alkaloid thaspine inhibits topoisomerase I activity using plasmid relaxation assays (Fig. **2A**) and show that the inhibitory effect is increased when the compound is pre-incubated with the enzyme before substrate addition (Fig. **2B**). These findings suggest that thaspine is able to interact directly with the enzyme alone, likely inhibiting the enzyme cleavage activity. This suggestion is confirmed by the cleavage experiments (Fig. **4**) that unambiguously demonstrate that thaspine acts as a cleavage inhibitor since the cleavage product is reduced to 50% and 13% when the reaction is carried out in presence of thaspine and after pre-incubation respectively (Fig. **4**).

However thaspine is also able to inhibit the religation process as indicated by the experiments reported in Fig. (**5**). The drug is then able to interact with the enzyme alone or with the enzyme-DNA cleavable complex, acting with a mechanism different by the CPT family drugs, the best characterized drugs for this class of enzymes [26-29]. CPT acts as a poison since it reversibly binds the cleavable complex, but not the enzyme alone, trapping it long enough to permit the collision with the replication fork. On the other hand thaspine is able to reversibly bind either the enzyme alone or the cleavable complex so acting either as a catalytic inhibitor or as a poison.

The docking of thaspine drug with topoisomerase I provides an atomistic explanation for the two inhibition effects. Docking of the drug in presence of DNA with the enzyme in the closed state shows that thaspine intercalates very well between the bases -1 and +1 at the nicked site (Fig. **6A**). This interaction explains the inhibition of the religation reaction, in fact stacking of the drug over the DNA bases sterically prevent the nucleophilic attack driven by the 5'-hydroxyl end to the phosphotyrosine bond and stabilizes the cleavable complex. Docking of the drug with the enzyme in the closed state in absence of DNA gives rise to a complex having an energy of -5 kcal/mol where the thaspine is arranged over the catalytic tyrosine (Fig. **6B**). However this interaction is very unlikely to occur since thaspine has been shown to inhibit the enzyme before its interaction with DNA, its inhibition power increasing upon an enzyme-drug pre-incubation (Figs. **2** and **3**). It is then likely that the drug interacts with the open state of the enzyme and in this case the docking simulations indicate that the drug has several interaction sites in proximity of the catalytic pentad with an energy of about -7 kcal/mol (Fig. **7A** and **7B**), providing an explanation for the cleavage inhibition.

Thaspine was previously found to inhibit both topoisomerase I and II *in vitro* [16]. The profile of gene expression observed in cells exposed to thaspine is similar both to that observed after ellipticine (a topoisomerase II inhibitor) and camptothecin (a topoisomerase I inhibitor) treatment [16]. The relative importance of the two different types of activities for the final biological effects are difficult to assess. It has been previously found that CEM/VM-1 cells, which express a mutated form of topoisomerase II and being resistant to inhibitors of this enzyme [30], are partially resistant to thaspine [16]. Together with the findings of the present study, available data suggest that inhibition of both types of topoisomerases are important for biological activity.

Thaspine was previously found have a very strong cytotoxic effect on 3-D multicellular tumor spheroids and induces apoptosis in human tumor xenografts *in vivo* [16]. Another attractive property of thaspine, adding to its potential as a future cancer therapeutic agent, is that the drug does not appear to be a substrate of drug transporters [16]. The cytotoxic activity of thaspine has been tested in the NCI_{60} cell line panel where it shows an average GI_{50} of 13 μM (www.dtp.nci.nih.gov), similar to the concentration required for topoisomerase I inhibition *in vitro*. Although this concentration is somewhat high, two leukemia cell lines, CCRF-CEM (human lymphoblastic leukemia) and K562 (chronic myeloic leukemia) shows a GI_{50} of 2 - 3 μM. Chemical modifications may be able to increase the cytotoxic effects of thaspine.

In conclusion our results indicate that thaspine is unambiguously targeting human topoisomerase IB sharing characteristics with poisons and catalytic inhibitors of topoisomerase I, being able to inhibit both the cleavage and the religation steps. Chemical modifications of the thaspine molecule may be able to confer specificity toward a selective inhibition of either of these two processes and may also increase the cytotoxic effects of the drug.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

ABBREVIATIONS

CPT	=	camptothecin
DMSO	=	Dimethyl sulfoxide

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