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Human topoisomerase IB is a target of a thiosemicarbazone copper(II) complex

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ABSTRACT

The human topoisomerase IB inhibition and the antiproliferative activity of 3-(4-bromophenyl)-1-pyridin-2-ylprop-2-en-1-one thiosemicarbazone HPyCT4BrPh alone and its copper(II) complex [Cu(PyCT4BrPh)Cl] was investigated. [Cu(PyCT4BrPh)Cl] inhibits both the DNA cleavage and religation step of the enzyme, whilst the ligand alone does not display any effect. In addition we show that coordination to copper(II) improves the cytotoxicity of HPyCT4BrPh against THP-1 leukemia and MCF-7 breast cancer cells. The data indicate that the copper(II) thiosemicarbazone complex may hit human topoisomerase IB and that metal coordination can be useful to improve cytotoxicity of this versatile class of compounds.

1. Introduction

Human topoisomerase IB (topo IB) is an essential enzyme, which controls the topological state of DNA during replication and transcription processes [1–3]. It has a considerable medical interest because it is the sole cellular target of camptothecins (CPT), a family of compounds used in cancer therapy [4,5], with topotecan and irinotecan approved for clinical use by the FDA [6]. CPT stabilizes the covalent intermediate enzyme-DNA, transforming topo IB in a poison for the cell [7,8]. Other bioactive compounds are able to inhibit topo IB, affecting binding to DNA and/or the chemistry of cleavage reaction [9–11].

Thiosemicarbazones and their metal complexes are a class of compounds exhibiting a wide pharmacological profile. α(N)-heterocyclic thiosemicarbazones have been shown to present anticancer activity [12]. 3-aminopyridine-2-carboxaldehyde thiosemicarbazone ("Triapine") was found to be effective against leukemia [13] and it has been evaluated in several clinical phase I and II trials [14]. The proposed mode of action of these compounds involves inhibition of ribonucleotide reductase, an enzyme that catalyzes the reduction of ribonucleotides to the corresponding 2′-deoxyribonucleotides during DNA synthesis. Thiosemicarbazones have been suggested to form an iron(III) complex within the cell, which is reduced to the corresponding iron(II) complex. The iron(II) complex may react with oxygen leading to the production of reactive oxygen species (ROS) able to inactivate the tyrosyl free radical that is present in the enzyme structure [15]. Direct reduction of the radical by the iron(II) complex has also been proposed [16].

Another established target for some α(N)-heterocyclic thiosemicarbazones is topoisomerase IIα (Topo IIα), an enzyme regulating DNA topology during cell division [17]. A series of α-(N)-heterocyclic carboxaldehyde thiosemicarbazones proved to have strong affinity for the enzyme, and their anti-proliferative activity was found to correlate with Topo IIα inhibition [18]. In addition, a family of 2-formylpyridine- and 2-acetylpyridine-derived thiosemicarbazones showed Topo IIα inhibition activity, which increased upon complexation with copper(II) with formation of square-planar complexes [19]. As far as topoisomerase I is

Abbreviations: topo IB, human topoisomerase IB; complex (**1**), [Cu(PyCT4BrPh)Cl]; CPT, camptothecin.

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concerned, enzyme inhibition was reported for zinc(II) complexes with polyhydroxobenzaldehyde derivatives [20].

Chalcones (1,3-diaryl-2-propen-1-ones) are a class of flavonoid natural products consisting of two aryl rings linked by an α,β-unsaturated ketone moiety, with widespread distribution in plants. These compounds possess a variety of biological activities as antioxidant, cytotoxic, anticancer, antimicrobial, antiprotozoal and anti-inflammatory agents [21]. Thiosemicarbazones with a great diversity of structural scaffolds have been investigated but there are not many reports in the literature on chalcone-derived thiosemicarbazones. In previous works a family of chalcone-based thiosemicarbazones and their zinc(II), gallium(III) [22] and copper(II) complexes were prepared [23] and it was shown that coordination to copper(II) significantly increased the cytotoxicity against Jurkat leukemia, against MDA-MB 231 (human breast carcinoma) and HCT-116 (human colorectal carcinoma) cancer cells [23].

In the present work, in order to further test the potentiality of chalcone-derived thiosemicarbazones as potential anticancer drug, the interactions of 3-(4-bromophenyl)-1-pyridin-2-ylprop-2-en-1-one thiosemicarbazone (HPyCT4BrPh) and its copper(II) complex [Cu(PyCT4BrPh)Cl] (Fig. 1), hereafter called complex (**1**) with topoisomerase IB were investigated. An evaluation of the cytotoxic effects of both compounds on THP-1 leukemia and MCF-7 breast cancer cells was performed as well. The results indicate that topoisomerase IB becomes the target of 3-(4-bromophenyl)-1-pyridin-2-ylprop-2-en-1-one thiosemicarbazone HPyCT4BrPh once it is coordinated to copper(II). In detail, complex (**1**) inhibits the enzyme in a dose dependent manner, acting primarily on the cleavage step of the topo IB catalytic cycle, although the religation rate is also perturbed. Pre-incubation of the enzyme-complex (**1**) before DNA addition improves the inhibitory effect of the thiosemicarbazone complex indicating that it acts as a topoisomerase inhibitor. On the other hand, the ligand alone does not show any inhibitory effect, highlighting the importance of metal coordination in providing a therapeutic function.

2. Materials and methods

2.1. Materials and compounds preparation

All commercial chemicals used as starting materials and reagents in this study were purchased from Sigma-Aldrich and were of reagent grade.

The synthesis of 3-(4-bromophenyl)-1-pyridin-2-ylprop-2-en-1-one thiosemicarbazone (HPyCT4BrPh) was performed as previously described [22]. Briefly a solution of NaOH 10% (7.5–12.5 ml) was added dropwise to a mixture of thiosemicarbazide (10 mmol) and 3-(4-bromophenyl)-1-pyridin-2-ylprop-2-en-1-one (10 mmol) in methanol (15 ml). The reaction mixture was kept under stirring for 1 h at room temperature. The resulting solid was filtered off, washed with water, and dried *in vacuum*. The synthesis of [Cu(PyCT4BrPh)Cl] complex was obtained as previously described [23] by refluxing a methanol solution of the ligand with copper(II) chloride in 1:1 ligand-to-metal molar ratio. The resulting solid was washed with methanol followed by diethylether and then dried *in vacuum*.

2.2. Topoisomerase IB production and purification

EKY3 yeast cells (ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1:TRP1, MATα) were transformed with YCpGAL1-e-wild-type plasmid, in which the human topoisomerase IB is expressed under the galactose inducible promoter [24]. The culture was grown in SC-uracil 2% dextrose over-night, diluted 1:100 in SC-uracil 2% raffinose and induced at an $OD_{595nm} = 1$ with 2% galactose for 6 h at 30 °C, 140 rpm. The cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol completed with protease inhibitors cocktail (Roche 1 836 153) supplemented with 0.1 mg/ml sodium bisulfite and 0.8 mg/ml sodium fluoride and disrupted using 425–600 μm diameter glass beads alternating vortexing for 30 s with 30 s on ice. The extract was loaded onto 1 ml ANTI-FLAG M2 Affinity Gel column equilibrated as described in the technical bulletin (Sigma). Topo IB interacts with the resin through the N-terminal sequence FLAG: DYKDDDY, and it is eluted with five column volumes of buffer containing 100 μg/ml FLAG peptide. Fractions were collected and glycerol was added to a final concentration of 40%. All preparations were stored at −20 °C [25].

2.3. DNA relaxation assay

To determine the units/μl of topo IB 1 μl of enzyme solution was diluted 3, 9, 27, 81-fold in 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) before addition of 0.5 μg of negatively supercoiled pBlueScript KSII(+) DNA in 30 μl of reaction volume. The reaction mixture was incubated at 37 °C for 30 min. One unit is defined as the amount of enzyme required to completely relax 0.5 μg of negative supercoiled plasmid DNA in 30 min at 37 °C.

One unit of topoisomerase IB was incubated with 0.5 μg of supercoiled DNA in 30 μl containing reaction buffer. To assess the effects of HPyCT4BrPh, complex (**1**) and $CuCl_2$ on topo IB activity different concentrations of each compound were added. Reactions

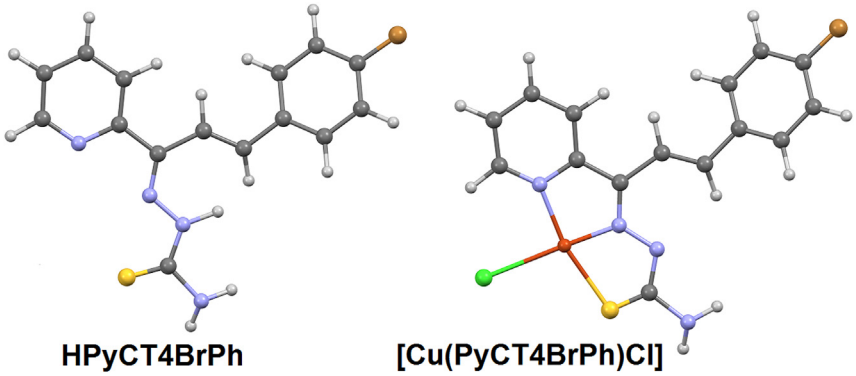

Fig. 1. Structures of HPyCT4BrPh and complex (**1**). The atoms are depicted as follows: C, dark gray; N, blue; H, light gray; Cl, green; Cu, orange; S, yellow; Br, brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were stopped with 0.5% SDS after 30 min at 37 °C. The samples were electrophoresed in a horizontal 1% agarose gel in TBE buffer (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 or DNA and inhibitor were pre-incubated at 37 °C for 5 min, prior of the addition of substrate or enzyme respectively. The mixture was then incubated at 37 °C for 15 min. Assays were performed at least three times and representative gels are shown [26].

2.4. Kinetics of cleavage

The oligonucleotide CL14 (5′-GAAAAAAGACTTAG-3′), radiolabelled with γ-^{32}ATP at its 5′ end, was annealed with a 2-fold molar excess of CP25 complementary strand (5′-TAAAAATTTTTC-TAAGTCTTTTTTC-3′) to produce the "suicide substrate", containing a partial duplex. 2.5 units of enzyme were incubated with 20 nM of suicide substrate in reaction buffer at 37 °C in the absence or in the presence of 12 μM complex (**1**). DMSO was added as a control. Before protein addition 5 μl of the reaction mixture were removed and used as the zero time point. At different time points 5 μl aliquots were removed and 0.5% SDS was added to stop the reaction. All the samples were precipitated in ethanol, digested with trypsin and analyzed using denaturing 7 M urea/20% 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 amount of the CL1 product obtained in each lane to the maximal CL1 level obtained at the longest time point in the absence of compound [27].

2.5. Religation kinetics

The CL14/CP25 substrate (20 nM), prepared as described above, was incubated with 2.5 units of topo IB for 30 min at 37 °C in reaction buffer to permit cleaved complex formation. DMSO or 12 μM complex (**1**) were added to the solution and religation reactions was initiated by adding a 200-fold molar excess of R11 oligonucleotide (5′-AGAAAAATTTT-3′) over the duplex CL14/CP25. At different time points 5 μl aliquots were removed and the reaction stopped with 0.5% SDS. All the samples were precipitated in ethanol, digested with trypsin and analyzed using denaturing 7 M urea/20% polyacrylamide gel electrophoresis. The experiment was replicated at least three times and a representative gel is shown. The percentage of the remaining covalent complex was determined by PhosphorImager and Image-Quant software and normalized relative to the total amount of radioactivity in each lane [28].

2.6. Electrophoretic mobility shift assay (EMSA)

The assay was performed using the CL25/CP25 full duplex DNA as a substrate, obtained by annealing radiolabelled CL25 (5′-GAAAAAAGACTTAGAAAAATTTTTA-3′) and the complementary strand CP25. The reactions were performed using the catalytically inactive mutant Tyr723Phe. The substrate (0.5 nM) was incubated with the enzyme in the presence of DMSO, 12 μM complex (**1**) or 50 μM camptothecin in reaction buffer at 37 °C for 10 min, in a final volume of 30 μl. The reactions were added of 3 μl of dye (0.125% Bromophenol Blue and 40% (v/v) glycerol) and loaded into 6% (v/v) polyacrylamide gels and electrophoresed at 40 V in TBE (12 mM Tris, 11.4 mM boric acid and 0.2 mM EDTA) at 4 °C for 4 h. As a control 12 μM complex (**1**) was incubated with substrate to show no change in electrophoretic mobility of DNA in the presence of compound [29].

Reaction products were visualized by PhosphorImage. Assay was replicated at least three times and only one representative gel is shown.

2.7. Docking predictions

Docking experiments were performed using Autodock 4.2 with MGLTools 1.5.6 [30] to prepare the structure of the ligand and the receptors. Calculations were made with the genetic algorithm [31] and for each experiment 250 runs were carried out. The structure of the receptor for the docking with the free protein was taken from the crystal structures 1A36 [32] and 1EJ9 [33], upon reconstruction of the missing residues as previously described [34]. Upon elimination of the DNA substrate the structure of the protein was used to carry out the docking with the free enzyme. Calculation with the covalent complex was carried out using the structure of the 1K4S binary complex [35] upon reconstruction of the linker domain as previously described [36]. Elimination of the 12 bases downstream of the cleavage site gave rise to the "suicide" protein-DNA covalent complex used for the last experiment (Supplementary Fig. S1). Coordinates of the complex (**1**) were obtained from the crystal structure downloaded from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. The bromine atom, which is not present in the solved structure, was added to the compound and the structure regularized using the program Sybyl (Tripos, St. Louis, MO) prior the completion of the docking.

The analysis of the contacts between the ligand and the receptor was performed using a modified version of the program g_mindist from the Gromacs 4.6 package [37], taking a threshold value of 3.5 Å. Images were obtained with VMD visualization package [38].

2.8. Evaluation of the cytotoxic effect against human tumor cell lines

Cells were seed at densities/well of 100,000 for THP-1 cells. Adherent MCF-7 cells were inoculated at 10,000 cells/well. The plates were pre-incubated for 24 h at 37 °C to allow adaptation of cells prior to the addition of the test compounds. Freshly prepared solutions of the compounds were screened at 10 μM. Subsequently, the plates were inoculated for 48 h in an atmosphere of 5% CO_2 and 100% relative humidity. Cell viability was estimated by measuring the rate of mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). All substances were dissolved in DMSO prior to dilution. IC_{50} values were determined over a range of ten-fold concentrations (100−0.001 μM). All compounds were tested in triplicate, in three independent experiments.

2.8.1. In vitro cell viability MTT assay

MTT is a tetrazolium salt that is reduced to purple formazan crystals mainly by mitochondrial succinate dehydrogenase and the color intensity of the formazan dye is correlated to the number of viable cells [39]. Briefly, at the end of the incubation of the cells with the different compounds, 20 μl of MTT solution (5 mg/ml in phosphate-buffered saline) were added to each well and incubated for 4 h. The supernatant was removed and 200 μl of 0.04 M HCl in isopropyl alcohol were added to dissolve the formazan crystals. The optical densities (OD) were evaluated in a spectrophotometer at 595 nm. Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as control (blank) and was treated in the same way as the drug-containing media. Results were expressed as percentage of cell proliferation, relative to cells incubated with the 0.5% DMSO control and were calculated as follows: viability (%) = (mean OD treated − mean OD background)/(mean OD untreated cultured, i.e. 0.5% DMSO − mean OD blank wells) x 100. Interactions of compounds and media were estimated on the basis of the variations

between drug-containing medium and drug-free medium to escape from false-positive or false-negative in relation to the control [40].

3. Results

3.1. Cytotoxic activity

The cytotoxic effects (IC_{50}) of HPyCT4BrPh and complex (**1**) against THP-1 leukemia and MCF-7 breast cancer cells are reported in Table 1. The complex (**1**) is more active than the free thiosemicarbazone as already reported for MDA-MB (breast cancer) and HCT-116 (colorectal cancer) tumor cells [41]. Upon coordination to copper(II), the cytotoxicity of HPyCT4BrPh increased 6-fold against THP-1 and 8-fold against MCF-7 cells.

As a control $CuCl_2$ at the same concentration was inactive as already shown for several leukemia and solid tumor cells [23]. These results indicate the importance of thiosemicarbazone metal coordination in providing cytotoxicity against these two tumor cell lines.

3.2. Topoisomerase IB inhibition

The inhibitory effect of the compounds on topoisomerase IB activity was tested through an agarose gel electrophoresis assay, comparing the different mobility among supercoiled DNA plasmid and the relaxed forms of DNA (topoisomers), produced by the enzyme activity. Circular plasmid DNA can exist in different topological conformations, that maintain the same sequence and molecular weight but that having a different degree of compactness migrate in different way i.e. supercoiled DNA migrates in an agarose gel faster than the relaxed topoisomers.

Topo IB efficiently relaxes the supercoiled substrate, as it can be appreciated from the disappearance of the band belonging to the supercoiled DNA substrate (SC) and the appearance of new bands corresponding to relaxed DNA topoisomers (Fig. 2A, compare lane1 and lane 3). The activity is hardly affected by the increase of thiosemicarbazone concentration up to 50 μM (Fig. 2A, lanes 4–10), on the contrary topo IB activity is significantly reduced when the enzyme is incubated with complex (**1**). The inhibition starts at a concentration of 0.75 μM and it is almost complete at 3 μM (Fig. 2B). As a control it has been observed that addition of $CuCl_2$ does not inhibit the enzyme up to a concentration of 200 μM, confirming that the effect detected at low complex (**1**) concentration cannot be due to the free metal (Fig. 2A and C).

3.3. Preincubation effect on topoIB activity

Complex (**1**) was pre-incubated with the enzyme or the DNA alone to verify the effect of pre-incubation in a time course relaxation inhibition assay (Fig. 3). Pre-incubating the enzyme with 0.75 μM complex (**1**), a concentration not sufficient to fully inhibit the enzyme (Fig. 3, lanes 6–9), before DNA addition results in full inhibition suggesting that the complex interacts with the enzyme alone (Fig. 3, lanes 10–13). Pre-incubation of complex (**1**) with the DNA substrate alone, before enzyme addition, does not increase the extent of inhibition observed in the absence of the pre-incubation (Fig. 3, lanes 14–17). As a control, the experiment in the presence

Table 1
Cytotoxic effect (IC_{50}) of compounds against THP-1 and MCF-7 cancer cells.

Compound	IC_{50} (μM)	
	THP-1	MCF-7
$HPyCT_4BrPh$	1.27 (±0.32)	1.23 (±0.25)
[Cu(PyCT4BrPh)Cl]	0.20 (±0.06)	0.16 (±0.06)

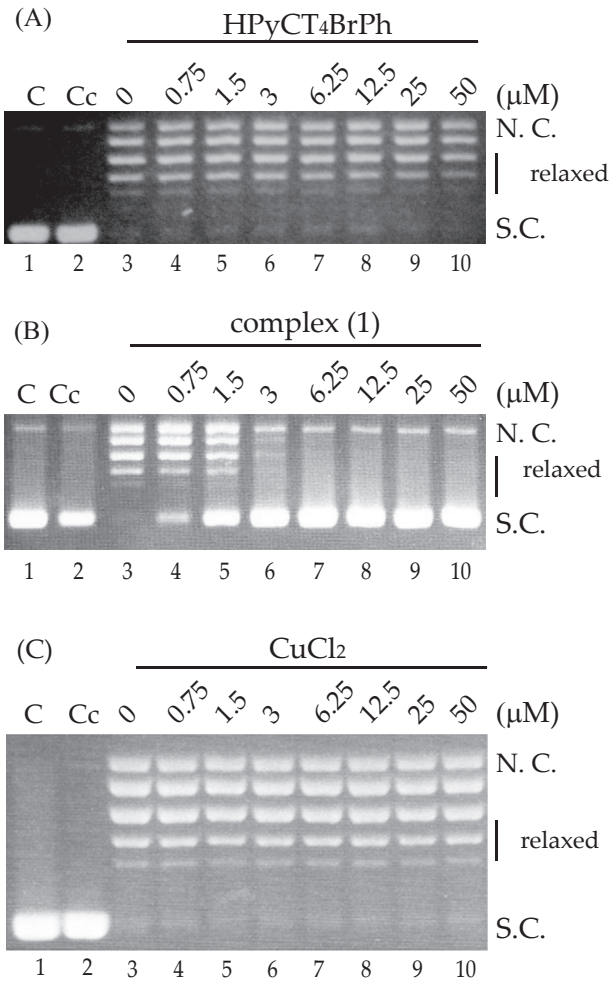

Fig. 2. Relaxation of negative supercoiled plasmid by topo IB in the presence of increasing concentrations of (A) HPyCT4BrPh, (B) complex (**1**), (C) $CuCl_2$ (lanes: 4–10). Lane 1: substrate. Lane 2: substrate plus an excess of (A) HPyCT4BrPh, (B) complex (**1**), (C) $CuCl_2$. Lane 3: substrate plus enzyme and DMSO. N.C.: nicked circular DNA. S.C.: supercoiled DNA.

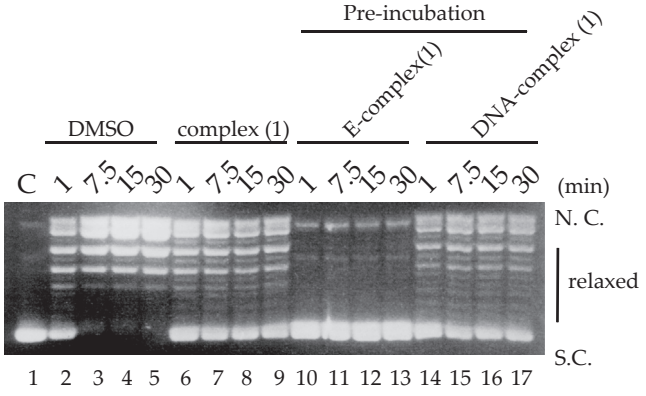

Fig. 3. Relaxation of supercoiled DNA by topo IB as a function of time in the presence of DMSO (lanes 2–5), 0.75 μM complex (**1**) (lanes 6–9), 5 min enzyme-complex (**1**) pre-incubation before DNA addition (lanes 10–13), 5 min DNA-complex (**1**) pre-incubation before enzyme addition (lanes 14–17). Lane 1: substrate. N.C.: nicked circular DNA. S.C.: supercoiled DNA.

of DMSO, at the same concentration used to dissolve complex (**1**), shows that the enzyme is fully active, confirming that the inhibition depends on the presence of the metal complex (Fig. 3, lanes 2–5).

3.4. Cleavage and religation rate

The cleavage step of the catalytic cycle of topo IB has been studied using the oligonucleotide CL14, radiolabeled at its 5'-end, annealed with the CP25 complementary strand to produce a duplex oligonucleotide with 11 nucleotides single strand extension. This substrate is named "suicide substrate" (Fig. 4A), because when the enzyme cuts it at its preferential site, indicated by an asterisk in Fig. 4A, the religation step is precluded, because the cleaved AG-3′oligonucleotide is too short to be religated. The enzyme remains covalently attached to the 12-oligonucleotide 3′-end forming the cleavage complex (CL1) that increases as a function of time, as shown in Fig. 4B. The cleavage reaction is fast in the absence of complex (**1**) and approximately 80% of the reaction occurs within 15 s (Fig. 4B and C). In the presence of 12 μM complex (**1**) the cleavage reaction is strongly inhibited and the quantity of cleaved substrate, normalized at the plateau value obtained in the absence of compound, is reduced to 20% even after 1 h, as shown in Fig. 4C.

The religation rate has been measured incubating the suicide substrate with an excess of enzyme to allow cleavage complex formation (Fig. 5A, lanes 2 and 9). A 200-fold molar excess of an R11 complementary oligonucleotide is then added permitting the enzyme to produce a full duplex oligonucleotide as final product. The urea-polyacrylamide gel electrophoresis of the products shows

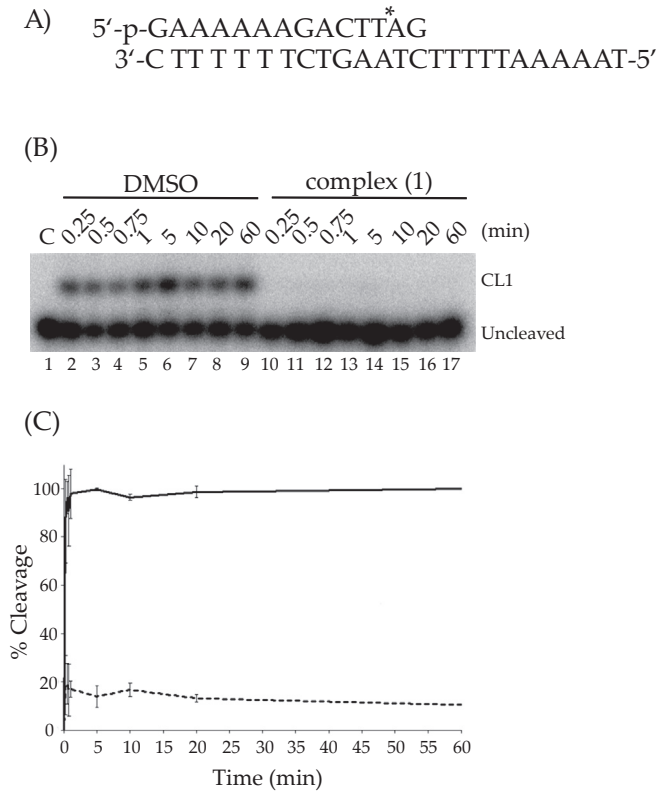

Fig. 4. **(A)** "Suicide substrate" for cleavage reaction. The asterisk indicates the preferential cleavage site. **(B)** Urea/polyacrylamide gel of cleavage products obtained in the absence (lanes 2–9) and in the presence of 12 μM complex (**1**) (lanes 10–17). Lane 1: substrate. CL1: cleavage complex. **(C)** % of cleavage plotted as a function of time in the absence (full line) and in the presence of 12 μM complex (**1**) (dotted line). The data reported are the average ± SD of three independent experiments.

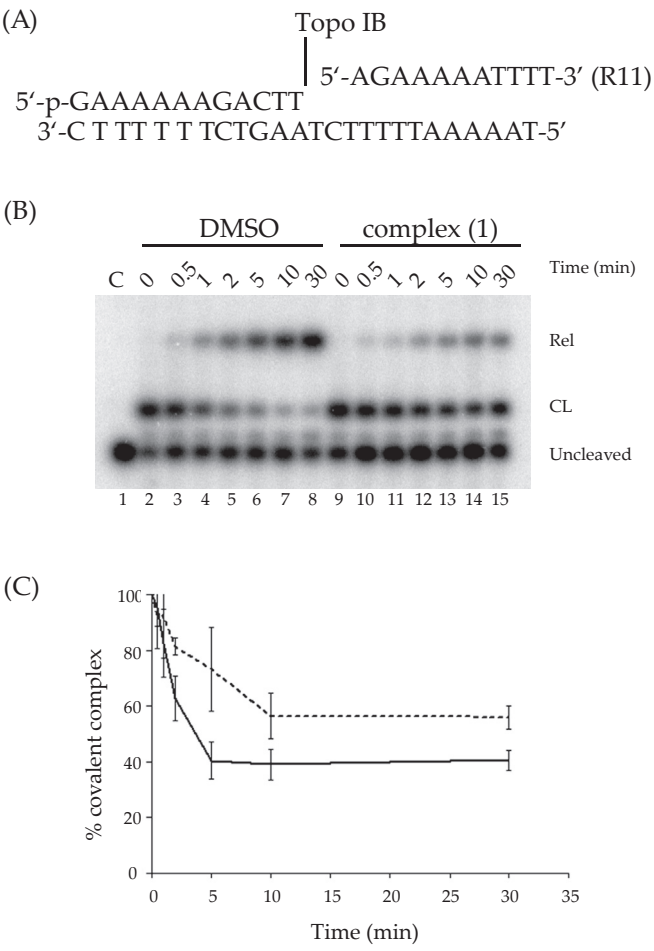

Fig. 5. **(A)** Schematic representation of religation reaction. **(B)** Urea/polyacrylamide gel of products of religation reaction performed by topo IB in the absence (lanes 2–8) and in the presence of 12 μM complex (**1**) (lanes 9–15). Lane 1: substrate. Rel: religated product. CL1: preferential cleavage site. Uncleaved: substrate. **(C)** % of remaining cleavage complex as a function of time in the absence (full line) and in the presence of complex (**1**) (dotted line). The data reported are the average ± SD of three independent experiments.

that the presence of 12 μM complex (**1**) reduces the rate of the reaction, indicating that the compound partially inhibits the religation step (Fig. 5B), as confirmed by the quantitative plot reported in Fig. 5C.

3.5. Electrophoresis mobility shift assay

An Electrophoretic Mobility Shift Assay (EMSA) was carried out using the catalytically inactive Tyr723Phe mutant. Using this mutant the effect of the compound on the chemistry of the cleavage reaction are eliminated, because the protein can not carry out the nucleophilic attack to the DNA phosphodiesteric bond, but it can still non-covalently bind to DNA. The assay was performed incubating a full duplex DNA radiolabeled substrate (CL25/CP25), containing the preferred cleavage site of topo IB, with the enzyme Tyr723Phe. After incubating the enzyme and DNA a retard on the electrophoretic mobility relative to the unbound DNA is observed due to the formation of an enzyme-DNA non covalent complex (Fig. 6, compare lanes 1 and 3). In the presence of 12 μM complex (**1**) the band corresponding to the non-covalent complex is not observed (Fig. 6, compare lanes 1 and 4), indicating that complex (**1**) does not permit the interaction between DNA and the enzyme

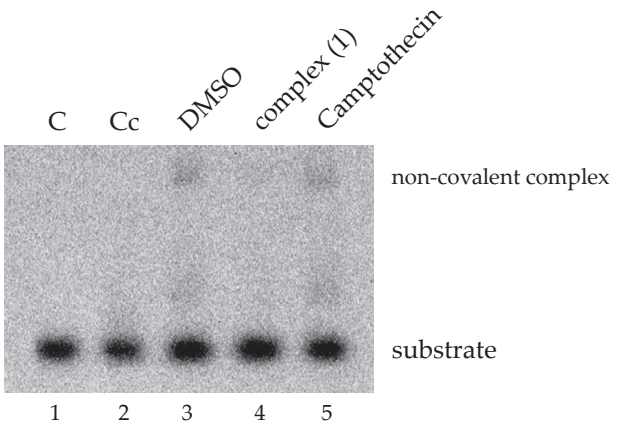

Fig. 6. Electrophoretic mobility shift assay. Lane 1: substrate. Lane 2: substrate in the presence of 12 μM complex (**1**). Substrate plus enzyme in the presence of DMSO (lane 3), 12 μM complex (**1**) (lane 4) and 50 μM CPT (lane 5).

and so providing an explanation for the absence of the cleavage activity in the presence of the metal complex.

3.6. Prediction of the complex (1) binding mode

Docking experiments between complex (**1**) and the protein alone or the covalent protein-DNA complex were carried-out. In the protein alone the compound is spread in several regions over the entire protein structure, with a binding energy ranging from −7.6 to −5.0 kcal/mol. In several of these sites the compound provokes steric hindrance for the DNA binding (Supplementary Fig. S1), thus providing an explanation for the inhibition of the cleavage activity (Fig. 4) and for the lack of the protein-DNA interaction reported by the EMSA experiments (Fig. 6). The docking with the covalent complex was performed using as a receptor the complex with a 22 bps DNA substrate cleaved at one strand or the complex containing the suicide substrate (Supplementary Fig. S2). The results are comparable. The most populated family of structures has a binding energy ranging from −7.3 to −6.7 kcal/mol and −8.0 to −7.0 kcal/mol for the covalent and suicide complexes respectively. In the first case the compounds lay at the interface between subdomain III and the linker domain (helix17-helix19) (Fig. 7A). Interestingly, complex (**1**) establishes contacts with Lys720 located on helix 20 that contains the catalytic tyrosine, and with His632 that is a residue of the catalytic pentad. In the case of the docking with the suicide complex, the compounds is located on helix 20 and interacts with Lys720 and Leu721 (Fig. 7B). Such interactions hinders the interaction with the substrate, providing an atomistic explanation for the religation inhibition (Fig. 5).

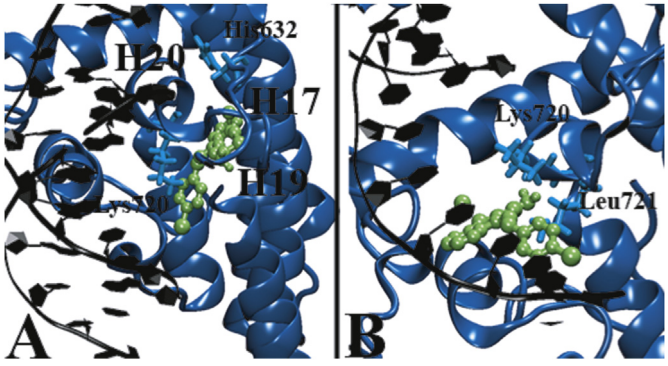

Fig. 7. Docking predictions of the complex (**1**) binding mode. Representative structure of the most populated family of structures in the docking with the (A) covalent complex and (B) suicide complex. In each panel the lateral chains of the residues interacting with the compound and the active site residue Tyr723 are shown in stick. The compound in the two panels is reported in lime and shown in Van der Walls representation. The helices are also defined as H17, H19 and H20.

4. Conclusions

HPyCT4BrPh and its copper(II) complex [Cu(PyCT4BrPh)Cl] (**1**), already known to be cytotoxic agents against HL60 leukemia cells, MDA-MB 231 and HCT-116 tumor cells, are shown to have cytotoxic effect also against THP-1 and MCF-7 cells. Coordination of the thiosemicarbazone to copper(II) improves the effect, making complex (**1**) more efficient than the parental compound (Table 1). In fact the ligand HPyCT4BrPh shows some cytotoxic activity, but upon coordination to copper(II) to form complex (**1**) the cytotoxic effect increases 6-fold against THP-1 leukemia cells and 8-fold against MCF-7 solid tumor cells (Table 1). This effect is due to complex (**1**) since addition of $CuCl_2$ does not induce any toxicity. Thiosemicarbazones have been suggested to exert their antineoplastic activity through several mechanisms such as Fe chelation, in this way being responsible for the iron impoverishment of the biological systems. They may also act through the redox cycling properties of the resulting Fe^{II}-thiosemicarbazones complex, inhibiting essential enzymes such as ribonucleotide reductase [42]. Other mechanisms are also described, such as inhibition of topoisomerase IIα, for which the binding of thiosemicarbazone to the ATP hydrolysis domain was shown to be responsible for enzyme inhibition [18].

Here we investigate the ability of HPyCT4BrPh and its copper(II) complex [Cu(PyCT4BrPh)Cl] (**1**) to inhibit human topoisomerase IB. For the first time we show that, after complexation with copper, the thiosemicarbazone becomes an efficient inhibitor of topoisomerase I. Complex (**1**) acts inhibiting the cleavage step of topo I catalytic cycle (Fig. 4). In particular complex (1) prevents enzyme-DNA binding, as demonstrated by the EMSA experiment reported in Fig. 6. This mechanism of action permits the classification of the copper complex as topo IB inhibitor although the compound is also able to partially affect the religation step (Fig. 5). Docking analyses support these results, showing that steric hindrance (Fig. 7) is responsible for DNA binding and religation step inhibition.

It is interesting that the topoisomerase inhibition activity is only observed upon copper(II) complexation highlighting the role of the metal in producing the right geometry to interact with the enzyme active site. The strategy of metal coordination is an interesting route that has been pursued for several ligands to confer them possible therapeutic effect and it is here shown to be successful also for the thiosemicarbazone ligand. This finding can increase the potential use of thiosemicarbazones as anticancer agents and confirms the important role of metal coordination in modulating the target of this class of compounds.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2016.07.009.

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