Università degli Studi di Roma *Tor Vergata*

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XXXI ciclo

Characterization of chimeric enzyme generated swapping human topoisomerase 1B N-terminal domain with *Plasmodium falciparum* counterpart and investigation of the interaction of human topoisomerase 1B with novel inhibitor derived from Antarctic invertebrates

Dottorato in Biochimica e Biologia Molecolare

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Characterization of chimeric enzyme generated swapping human topoisomerase 1B N-terminal domain with *Plasmodium falciparum* counterpart and investigation of the interaction of human topoisomerase 1B with novel inhibitor derived from Antarctic invertebrates

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Bini Chhetri Soren

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Coordinator: **Prof. Gerry Melino**

Dedicated to

My Parents,

My Sister

Family & Friends

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I would like to express my deepest gratitude to all the people who were part of this wonderful experience. In this period, I have leant a lot not only at scientific level but personal level as well.

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ABSTRACT

Plasmodium falciparum is a protozoan parasite that causes malaria, one of the most frequent acquired red blood cell diseases worldwide. The emergence of multi drug resistant strains has increased the need to identify new molecular targets for anti-malarial therapy. DNA topoisomerases may be considered possible candidates, due to their important role in cellular activities, including DNA replication. DNA topoisomerase 1 relaxes supercoiled DNA by a transient DNA strand breakage, rotation, and religation. The 3D structure of the human topoisomerase 1- DNA complex identifies two domains forming a conserved protein clamp, tightly wrapped around the DNA duplex and an extended coiled-coil linker domain that appropriately positions the C-terminal active site tyrosine against the core to form the catalytic pocket. The N-terminal domain of human topoisomerase 1 is the only part of the enzyme that is still not crystallized and the function of this domain is not fully known. It has been suggested that the N-terminal domain of human topoisomerase 1B contribute little to the enzyme activity but recent studies show that this domain significantly modulates in vitro DNA relaxation.

The *Plasmodium falciparum* topoisomerase 1 contains a shorter N-terminal domain, compared to the human homologue. This finding pushed us in exploiting its structural and functional properties in enzyme activity by swapping the human N-terminal domain with the corresponding one from *Plasmodium falciparum*. The chimeric enzyme has been functionally characterized in this thesis opening a route for the identification of new species selective drugs.

In this thesis, I have also screened novel inhibitors of human topoisomerase 1B derived from Antarctic sponge *Artemisina plumosa*. The geographical and evolutionary history of the Antarctica results in a unique and isolated ecosystem rich in different kinds of organisms that can develop natural products and metabolites having distinctive and specific biological activities. Analysis of these metabolites can be helpful in identifying new and novel drugs having possible biological activities such as cytotoxic, inhibitory, antiparasitic and so on. In the present study, we screened and identified a

new natural compound derived from Antarctic sponge *Artemisina plumosa* acting as an inhibitor of the human topoisomerase 1B activity. This compound will be tested in future also on the chimeric enzyme to better understand how to develop specific drugs for different topoisomerase 1 species.

1. INTRODUCTION

1.1 DNA Topology

DNA (deoxyribonucleic acid) is the hereditary material which encodes all the genetic information in humans and almost all other organisms. DNA is mostly located in the nucleus but some amount of DNA is also present in mitochondria. It is composed of pairs of repeated nucleotides and each nucleotide consists of a phosphate group, a 2'-deoxyribose sugar, and one of the four bases: Adenine (A), Thymine (T), Guanine (G) or Cytosine (C). These nucleotides are arranged in the form of a twisted ladder in which two strands are wound around one another thereby, forming the famous 'double helix' structure. The alternating sugar and phosphate groups forms the backbone of the ladder and pairs of bases forms the rungs of the ladder. These bases always pair either A with T or G with C where AT rung is formed by 2 hydrogen bonds between A and T, while the stronger GC rung consists of 3 hydrogen bonds between G and C.

There are different kinds of DNA structures: A, B, C and Z. A, B and C have the right-handed conformations whereas, Z is the only left-handed. The most common DNA structure is form B and its structure was famously pieced together by Francis Crick and James Watson, based on the X-ray images detected by Rosalind Franklin (Watson and Crick, 1953). The DNA B has a diameter of about 20Å and a pitch of 35.4 Å; the rotation angle between the pairs of adjacent bases along the axis is 34.6°, which are necessary for 10.4 pair bases as the helix makes a complete turn of 360°.

The topological aspects of the DNA structure arise mainly due to the fact that the two DNA strands are repeatedly twisted and intertwined. All the major biological process requires untangling of these two strands which proves to be difficult. In case of linear DNA in a solution, untangling could be possible due to the free rotating ends of the DNA; however, free rotation is either limited or completely prohibited in all natural DNAs (Merkin, 2001). In such kind of circumstances, the topological properties of DNA can be defined as those that cannot be changed without breaking one or both strands of the DNA double helix (Ketron andOsheroff, 2014). The topological relationships in DNA can be categorized into two (Figure 1):

- between the two strands of the double helix (i.e., supercoiling)
- between different segments of duplex DNA (i.e., tangling and knotting)

The classical Watson-Crick Model describes doubled stranded DNA that is free from torsional stress as "relaxed", hence, DNA that is under torsional stress is termed as "supercoiled". DNA molecules that are underwound are called negatively supercoiled [(-)SC] and molecules that are overwound are positively supercoiled [(+)SC]. Separation of two strands of the double helix is very important for the genetic information to be replicated or expressed; therefore, negative and positive supercoiling have great implications for DNA function.

Tangles and knots have an adverse affect on a variety of essential processes involving nucleic acids. Formation of knots makes it impossible for the separation of the double helix and can be lethal to the cells if they are not solved.

The fundamental parameters determining the topological state of DNA are twist (Tw), writhe (Wr), and linking number (Lk) (Bates and Maxwell 2005). The total number of double helical turns in a given segment of DNA is termed as twist whereas writhe represents the number of times the double helix crosses itself if the molecule is projected in two dimensions. The linking number is equivalent to the sum of twist (Tw) and writhe (Wr) and represented by

$$Lk = Tw + Wr$$

The change in the linking number (ΔL), is the number of turns in the molecule (L) minus the number of turns in the relaxed molecule L0.

$$\Delta L = L - L0$$

If the DNA is negatively supercoiled $\Delta L < 0$ which implies that the DNA is underwound. Negative supercoils favour local unwinding of the DNA, allowing biological processes such as transcription, DNA replication, and recombination to occur. Linking number is always an integer number and it

cannot be changed by any deformation of the DNA strand. The only way to change the linking number is by introducing a break in one or both DNA strands and rotating the two DNA strands relative to each other and seals the break.

The understanding of DNA topology raises an important question that if strand separation in a topological domain is so difficult than how does the intracellular DNA functions? This problem is solved by a class of enzymes known as DNA topoiomerases which was discovered in 1971 by J.C. Wang (Wang et al., 1971).

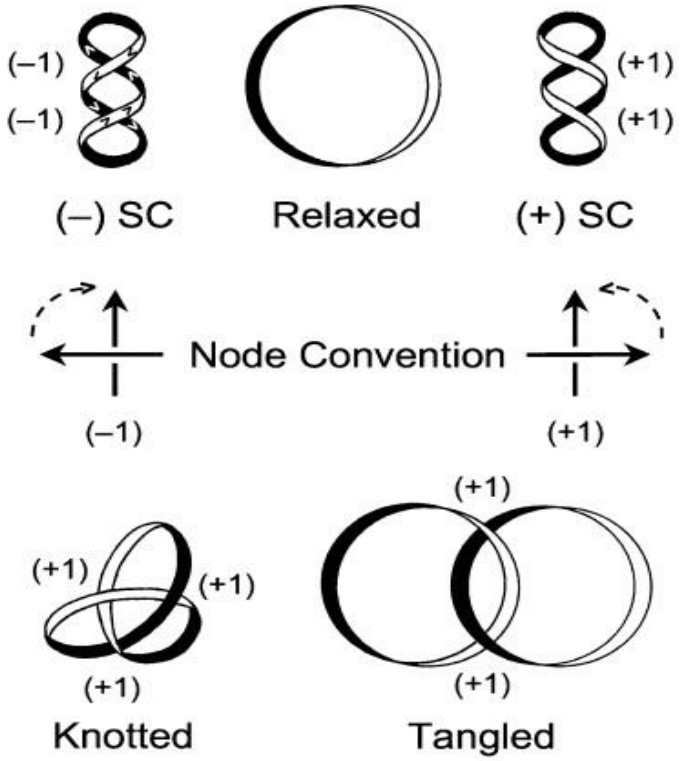


Figure 1: Topological relationship within DNA (Adapted from Ketron and Osheroff, 2014)

1.2 DNA topoisomerase

DNA topoisomerases are enzyme responsible for managing the topological state of the DNA inside the cell (Champoux et al., 2001). Regulation of DNA topology is very important for cell survival therefore these enzymes are encoded by all known species. DNA topoisomerases regulates the topological state either by passing one strand of the DNA through a break in the opposing strand (type I subfamily) or by passing a region of duplex from the same or a different molecule through a double-stranded gap generated in a DNA (type II subfamily) (Cretaio et al., 2007).

The basic need for DNA topoisomerase arises due to the double helical structure of the DNA. The majority of biological process requires separation of DNA either temporarily (e.g. transcription or recombination) or permanently (replication). These separation leads to topological problems which must be solved by DNA topoisomerase. Recently, there has been an immense interest in the study of toposiomerase mainly due to two reasons (Champoux et al., 2001). Firstly, a wide range of topoisomerase-targeted drugs, including antimicrobials and anticancer chemotherapeutics, have been identified (Cuya et al., 2017), and some of these drugs are currently in clinical use. Secondly, the crystal structures of a number of topoisomerase fragments have been published in the past few years providing key information on the function of this molecular machine functions (Champoux et al., 2001).

DNA topoisomerase are classified based on whether they introduce a single stranded or a double stranded break in the DNA (Figure 2) (Cretaio et al., 2007). Type 1 topoisomerase (Top1) catalyzes the transient break of only one of the double strand (Forterre et al., 2006). Type 1 are further divided in type 1A and type 1B on the basis of the polarity of DNA strand cleavage. Type 1a forms a transient 5′-phospho-tyrosine covalent intermediate and releases a free 3′-OH strand whereas Type 1b forms a 3′-phospho-tyrosine covalent intermediate and releases a free 5′-OH strand. The majority of type 1 topoisomerases are monomeric. Type 2 topoisomerases cleave both strand to generate a staggered double strand break. Based on the structural consideration they are sub divided into two types 2a and 2b. All type 2

topoisomerases are able to catenate /decatenate and knot/ unknot circular duplex DNA and relaxes positively as well as negatively supercoiled DNA. Type 2 topoisomerases are ATP dependent and are essential for segregation of the chromosomal DNA after DNA replication and before cell division (Forterre et al., 2006).

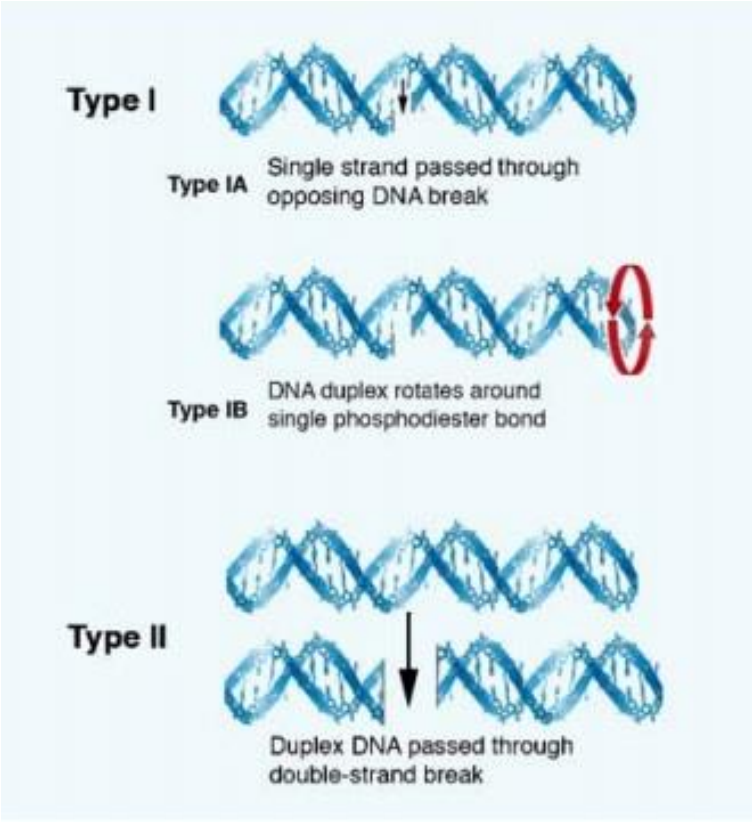


Figure 2. Types of DNA topoisomerases. (Adapted from Leppard et al., 2005)

1.3 Human topoisomerase 1B

The members of the type 1B subfamily of topoisomerases 1 share no sequence or structural homology with other known topoisomerases and are functionally different from the members of the type IA subfamily. Unlike the type IA enzymes, the type IB subfamily members can relax both positive and negative supercoils, and relaxation goes to completion. The type IB topoisomerases form a covalent intermediate in which the active site tyrosine is covalently attached to the 3'-phosphate end of the cleaved strand rather than to the 5'-phosphate end as for the type IA enzymes. These enzymes do not need divalent metal ion or cofactor (high energy) but its activity is influenced by the presence of metal ions like Mg^{2+} and Ca^{2+}. Based on sequence comparison it is possible to infer that the important features of human topoisomerase enzyme are maintained in all topoisomerase 1 from other eukaryotic species. The humans encode for six topoisomerases (Leppard et al., 2005) (table 1), out of these six, human DNA topoisomerase 1 is the best studied among the cellular topoisomerase enzyme (Champoux et al., 2001).

Table 1: Different topoisomerases encoded by Humans

Enzyme	Type	Cellular roles
Topoisomerase 1	1B	Replication, transcription, chromatin remodeling
mtTopoisomerase 1	1B	Mitochondrial replication, transcription
Topoisomerase II α , β	2A	Replication, chromosome segregation
Topoisomerase III α , β	1A	Recombination, repair

(Adapted from Leppard et al., 2005)

The structure of the human topoisomerase 1B has been deduced based on different studies such as conservation of sequence, sensitivity to limited proteolysis, hydrodynamic properties, and fragment reconstitution experiments. These studies indicated that human enzyme is composed by 765 amino acid residues and subdivided into four distinct domains namely the N-terminal (1-214), the core (215-635), the linker (636-712) and the C-terminal domain (713-765) (Figure 3).

The N-terminal 214 amino acids of the human enzyme constitute a hydrophilic, unstructured, and highly protease-sensitive region of the protein (Stewart et al., 1996). N-terminal domain contains four nuclear localization signals and sites for interaction with other cellular proteins, including proteins like nucleolin, SV40 T-antigen, certain transcription factors, p53, and the WRN protein (Bharti et al., 1996; Albor et al., 1998; Simmons et al., 1996). The N-terminal domain is followed by core domain which is a highly conserved domain and contains 421 amino acid having all of the catalytic residues but the active site tyrosine (Redinbo et al., 1998). A protease-sensitive and poorly conserved linker domain comprising 77 amino acids connects the core domain to the 53 amino acids of the C-terminal domain. An active form of the enzyme can be reconstituted by mixing together fragments corresponding to the core domain (residues 175 to 659) and the C-terminal domain (residues 713 to 765), showing that the linker is dispensable for relaxation activity in vitro. The active site Tyr723 is located the C-terminal domain.

The crystal structure of several forms of the human topoisomerase enzyme have been determined showing both non-covalently and covalently DNA binding (Redinbo et al., 1998; Redinbo et al., 2000; Stewart et al., 1998). The crystal structure was obtained with an N-terminal truncated active form of the protein in which the first 174 amino acids are missing. The X-ray density was interpretable only from residue 215, therefore the entire N-terminal domain is still not crystallized. In the year 2000, Redinbo et al determined the crystal structure of a form of the enzyme where the structure extends back to amino acid residue 203.

The N-terminal domain has a molecular weight of ~27 KDa. This domain is poorly conserved, highly charged, and protease-sensitive. The N-terminal domain is important for the strand rotation and relaxation in vivo (Frøhlich et al., 2004; Lisby et al., 2001). It contains five nuclear localization signals responsible for subcellular localization of the enzyme (Mo et al., 2000). The N-terminal domain is followed by core domain which is highly conserved, resistant to proteolysis and contains all the residues forming the active site Arg488, Lys532, Arg590, His632 excluding the catalytic tyrosine 723 (Figure 4a, black bars).

Based on the structure the core domain is further divided into three subdomains. Subdomain I (aa 215 – aa 232 and aa 320 – aa 433) and II (aa 233 – aa 319) forms a "cap" region containing a pair of α-helices called the "nose cone" (Figure 4, b, left). The Core subdomain III (aa 434 – aa 635) forms the "cap" region together with the C-terminal domain, which contains the catalytic tyrosine (Schoeffler et al., 2008; Wang et al., 2015). Human topoisomerase 1B (hTop1B) clamps around the DNA permitting the interaction of lip1 and lip2, belonging to core subdomain I and III, respectively (Figure 4, b, right). Interaction of the lips occurs through a non-covalent interaction between the carboxylic lateral group of Glu497 and the side-chain amino group of Lys369. Opening and closing of the protein clamp during DNA binding and release must involve the breaking of this interaction between the lips and the lifting of the cap away from the base.

The linker domain is protease sensitive and poorly conserved. It has a molecular weight of ~5 KDa and it is involved in the formation of "coiled-coil" structure that protrudes from the globular shape of the enzyme (Figure 4, b) (Redinbo et al., 1998). The linker plays a major role in modulating the DNA cleavage–religation equilibrium (Stewart et al., 1997), and it is supposed to act as a "brake" downstream of the cleavage site, during the rotation of the DNA in relaxation reaction (Stewart et al., 1999). The C-terminal domain is highly conserved and has a molecular weight of ~7 KDa. It is organized into five short α-helices, containing the catalytic tyrosine (Tyr723) (Figure 4, b). Tyr723 is placed on the loop connecting α-helices 20

to 21 and its mutation to phenylalanine inactivates the enzyme (Redinbo et al., 1998).

A hinge that allows the clamp to open or close around DNA is likely located at the α-helix linking upper and lower parts (Figure 4, b, right). Opposite to the hinge there are two loops referred to as the "lips" that interact one with the other to bring the cap close to the base of the protein (Figure 4, b, right).

The C-terminal domain is connected to core sub domain III via the linker domain. Study of the structure lead to the understanding of the human topoisomerase 1B (hTop1) catalytic mechanism and the term "controlled rotation" was coined to indicate that the linker domain likely hinders or slows down the rotation reaction (Stewart et al., 1998).

The catalytic cycle of human Top1B is characterized by five steps (Figure 5):
(1) Non covalent DNA binding by the enzyme (Figure 5A);
(2) Covalent binding through a transesterification reaction between the hydroxyl group of a tyrosine residue and the 3' end of the cleaved strand (Figure 5B-C);
(3) Rotation of the 5' end around the intact strand, to relax the supercoiled DNA using the energy stored in the DNA–TopIB complex (Figure 5D);
(4) Religation of scissile strand (Figure 5 E-F);
(5) Release of the relaxed DNA (Figure 5 G)

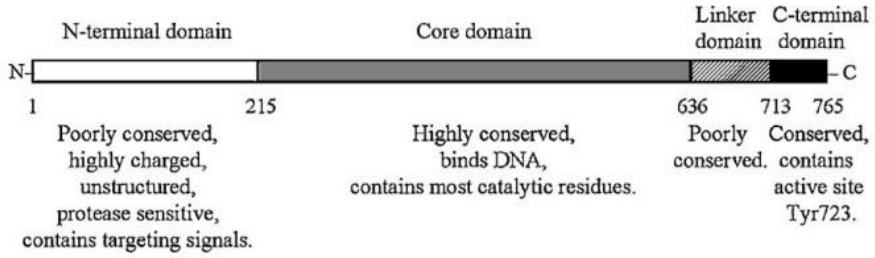


Figure 3. Domain structure of human topoisomerase 1b (Adapted from Champoux et al., 2001)

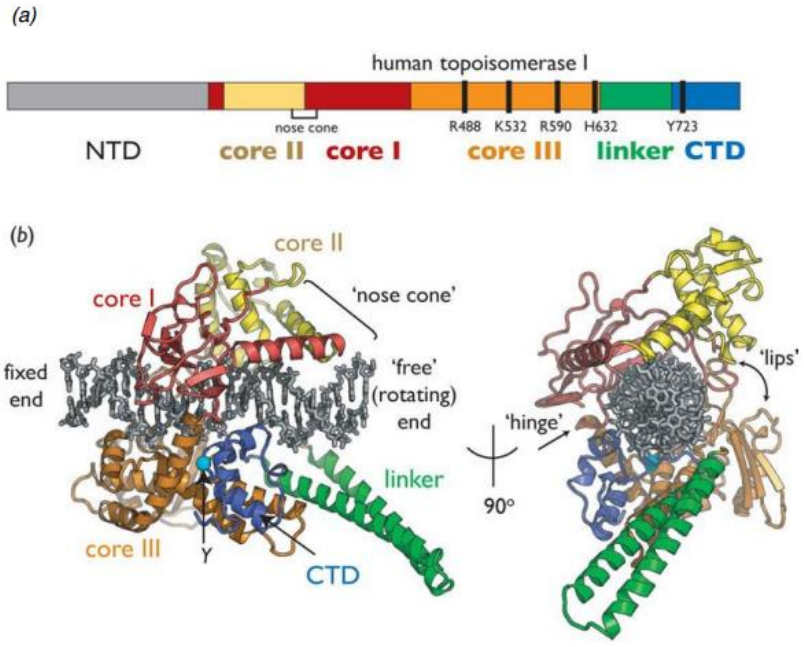


Figure 4. Type IB topoisomerase organization and structure. (a) The primary domain structures of human topoisomerase 1B. Key catalytic residues are indicated by black bars; NTD: N-terminal domain, CTD: C-terminal domain. (b) Two views of the structure of human topoisomerase I bound to DNA. Domains are colored as in (a); the catalytic tyrosine (Y) is shown as a cyan sphere. DNA is colored gray. The covalently bound (fixed) and unbound(rotating) ends are indicated. The lips and hinge region are labelled in the orthogonal view at right. (Structures are available on the protein data bank: http://www.rcsb.org; identificationnumbers 1A36are.) (Adapted from Schoeffler et al., 2008)

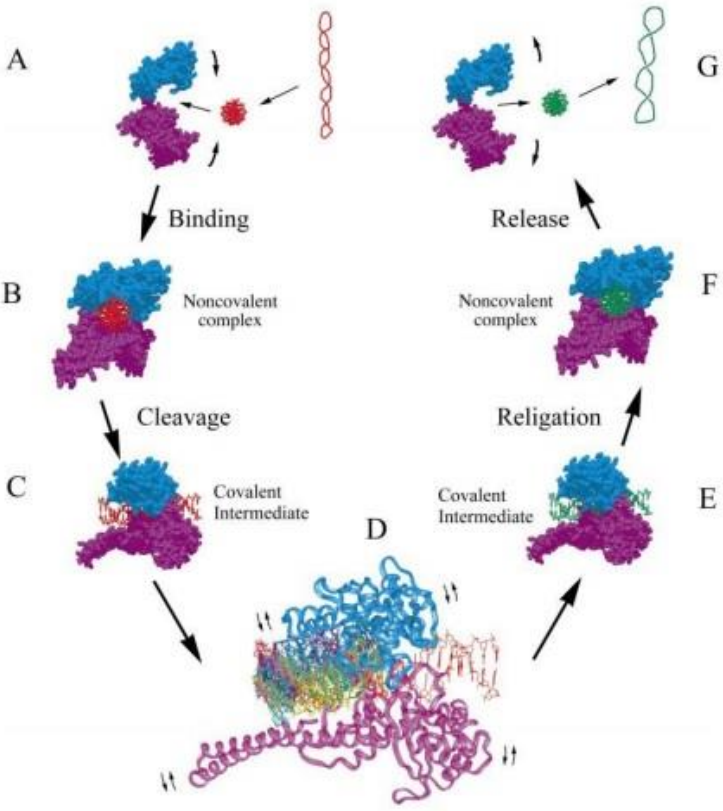


Figure 5. The controlled rotation mechanism of hTop1B. The highly negatively supercoiled substrate (red with right handed writhe) becomes partially relaxed through steps (A) to (G) and is converted to the less supercoiled state depicted in green. Because the shape of the enzyme is complementary to but not always in direct contact with the surface of the substrate DNA, small rocking movements (small arrows) may be allowed during the events of controlled rotation. (Adapted from Stewart et al., 1998)

1.4 DNA topoisomerase from protozoan parasite *Plasmodium falciparum*

The phylum *Apicomplexa* includes a large group of protozoan parasites responsible for a wide range of animal and human diseases. One of them, *Plasmodium falciparum*, is the causative agent of human malaria. Malaria is one of the most important worldwide health issues given that more than 40 per cent of world population is affected by this disease. It is endemic in over 108 countries inhabited by roughly 3 billion people. In the year 2010, it caused an estimated 216 million cases and 655000 deaths worldwide. More than 85% of malaria cases and 90% of malaria deaths occur in sub-Saharan Africa, mainly in young children (younger than 5 years) (White et al., 2017).

Plasmodium falciparum (the most dangerous species of the genus *Plasmodium*) undergoes asexual multiplication within erythrocytes, causing anemia, fever, chills, nausea, a flu-like illness and, in severe cases, coma and death (Carter and Mendis, 2002). The infectious cycle begins when a *Plasmodium* bearing mosquito takes a blood meal from a susceptible host. Sporozoites which have matured in the midgut of the mosquito are injected from the salivary glands into the host during the bite, where they migrate to the liver to form hepatic stages. Hepatic stages mature to merozoites which are released into the bloodstream and initiate the main pathogenic cycle (Miller *et al*., 2002). Each merozoite successfully invading a red blood cell passes through three distinct stages of development: the relatively inert ring stage, a metabolically active trophozoite stage and the segmenting schizont stage. The parasite divides four times during schizogeny, yielding on average 16 new merozoites (Bannister *etal*., 2000) and thus an exponential amplifcation of the parasite population, with one intraerythrocytic cycle completed in 48 hours (Bannister *etal*., 2000) (Figure 6)

Plasmodium falciparum contains two endosymbiotic organelles having their own mitochondrial and apicoplast DNA. Apicoplast is an attractive target for drug testing because in addition to harboring singular metabolic pathways absent in the host, it also has its own transcription and translation machinery of bacterial origin. Accordingly, the parasite has an interesting mixture of enzymes to unwind DNA from eukaryotic and prokaryotic origins, such as type I and type II eukaryotic topoisomerases (Table 2).

These enzymes are targeted by camptothecin and etoposide, respectively, two natural drugs whose semisynthetic derivatives are currently used in cancer chemotherapy. On the other hand, DNA gyrase is a bacterial-borne type II DNA topoisomerase that operates within the apicoplast and is effectively targeted by bacterial antibiotics like fluoroquinolones and aminocoumarins (Garcia-Estrada *et al*., 2010). In the year 1995, Tosh &Kilbey identified the gene encoding *Plasmodium falciparum* topoisomerase 1B (PfTop1B) which is a monomeric protein of 104 kDa containing 839 amino acids. The enzyme was found to be located on plasmodial chromosome number 5. This enzyme structurally resembles other topoisomerases, maintaining 42 per cent homology with the human enzyme (Garcia-Estrada *et al*., 2010). The active tyrosine is present at position 798. Sequence analysis showed the presence of two extensive tracts of additional amino acids within the core domain one is of 29-34 amino acids and another of 79 amino acids, the function of these additional amino acids remains unknown (Cheesman 2000). Studies show that PfTop1b is developmentally regulated during the different stages of *Plasmodium falciparum* life cycle (Tosh et al., 1999). Northern analyses show that PfTop1B gene promoter is inactive during the ring forms and becomes active during the asexual intra-erythrocytic cycle. High levels of PfTopIB mRNA are found during the throphozoite stage, but not in schizonts. Arnò et al. demonstrate an interesting finding in which human/plasmodial hybrid enzyme, generated by swapping the human topoisomerase IB linker domain with the corresponding domain of the *Plasmodium falciparum* topoisomerase enzyme displays a relaxation activity comparable to the human enzyme, but it is characterized by a much faster religation rate. The hybrid enzyme is also camptothecin resistant.

Table 2: Actual and putative topoisomerase enzymes in the genome of *P.falciparum*3D7 as annotated in PlasmoDB (Aurrecoechea et al., 2009)

Accession	Chromosome	Annotation
PFE0520c	5	TopoI
PF10_0412	10	Topoisomerase (putative)
PFL0825c	12	Conserved plasmodium protein unknown function
PF11_0477	11	CCAAT-box DNA binding subunit B
PFL1120c	12	DNA gyrase subunit A
PFL1915w	12	DNA gyrase subunit B
PF13_0251	13	DNA topoisomerase III (putative)
PF14_0316	14	DNA topoisomerase II putative

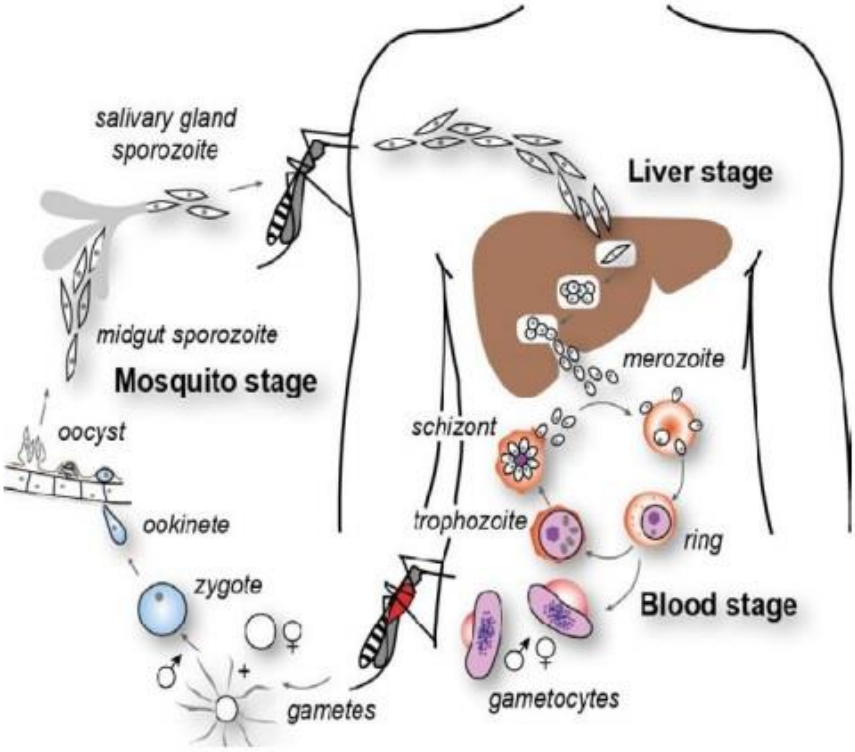


Figure 6. Life cycle of *P falciparum* parasite from mosquito bite to retransmission. Insets show merozoite invasion into red blood cells (RBCs) and sequestration in placental and epithelial tissue (Cowman AF *et al*., 2012).

1.5 N-terminal domain of human topoisomerase 1B

The N-terminal domain of human topoisomerase 1 (hTop1) is the only part of the enzyme that is still not crystallized and the function of this domain remains unknown (Frøhlich et al., 2004). In the course of evolution, the N-terminal domain has been preserved as a highly basic region in all cellular type 1B, although they differ in amino acid sequence (Lisby et al., 2001). This domain has shown to be responsible for a number of nuclear localization sequences (Table 3), phosphorylation sites and protein interaction (Alsner et al., 1992; Staron et al., 1998; Samuels et al., 1994). It has long been thought that N-terminal domain of human topoisomerase 1B contribute little to the enzyme activity but Lisby and co-worker showed that this domain significantly modulates in vitro DNA relaxation (Lisby et al. 2001).

Table 3. N-terminal domain of human topoisomerase 1 and their nuclear localization signals

Nuclear Localization Signals (NLS)	Amino acids	Sequence
NLS-I	59 aa -65 aa	KKHKEKE
NLS-II	150 aa -156 aa	KKIKTED
NLS-III	174 aa -180 aa	KKPKNKD
NLS-IV	192aa -198 aa	KKKPKKE

A number of observations suggest that phosphorylation can modulate enzyme activity and CPT sensitivity of hTop1. Hackbarth JS *et al.* identified four hTop1 residues that are phosphorlayated in intact cells: Ser10, Ser21, Ser112 and Ser394 suggesting that two residues Ser112 and Ser94 were phophorylated*in-vitro* by Cdk1. The phopshorylation of these four sites did not alter hTop1B localization or protein-protein interactions. Ser21 phosphorylation enhanced hTop1B relaxation activity *in-vitro* and CPT-induced stabilization of cleavage complexes in cells (Hackbarth JS *et al.*, 2008). Studies suggest that residues 191 -206 are required for DNA binding and enzyme processivity (Laco GS and Pommier Y., 2008). The close interaction of Trp-205 to residues in the flexible hinge region have suggested that Trp-205 plays an important role in controlling the motion within the hinge region involved in control of rotation (Frohlich RF *et al.,* 2004). Interestingly alignment of the human and plasmodium enzyme indicates that this tryptophan is also present in the pfTop1. However, the pf-N-terminal amino acids composition is quite variable as compared to hTop1 N-terminal since alignment of the two sequences shows that pfTop1 shares 24% identity and 50% similarity with the hTop1 topoisomerase (Figure 7).

Figure 7. Alignment of the sequence of the N-terminal domain of the human topoisomerase IB and *Plasmodium falciparum* topoisomerase I. Conserved residues are represented in blue color.

1.6 Topoisomerase 1 inhibitors

HTop1 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 hTop1 are divided into two classes: poisons and catalytic inhibitors. Poisons include camptothecin (CPT), a plant alkaloid first identified from the chinese tree, *Camptotheca acuminata* (Wall and Wani, 1995). CPT acts as an interfacial uncompetitive inhibitor (Pommier, 2009), reversibly trapping Top1 -DNA covalent complex (Top1 cc) (Figure 8) (Dexheimer and Pommier, 2008; Hsiang *et al*., 1985) by binding at the enzyme-DNA interface and stacking between the base pairs flanking the cleavage site, thus inducing cell death (Figure 8C) (Capranico *et al*., 1990; Jaxel*et al*., 1991; Pommier, 2009). In this ternary drug–enzyme–DNA complex, religation is prevented by the CPT-induced misalignment of the 5′-hydroxyl group and the scissile tyrosine–DNA bond. Although, these protein-linked DNA lesions are not toxic in themselves but they induce the collision of DNA replication forks with the ternary complexes, or with the positively supercoiled DNA domains induced by these complexes, producing irreversible DNA lesions and double-strand breaks that ultimately lead to cell death (Cuya et al., 2017). This ternary complex is also stabilized by additional bonds that would link the aromatic structure of the drug to the enzyme (Pommier, 2009), involving several aminoacidic residues such as Asn722.

Human leukemia cells selected for CPT -resistance showed an Asn722Ser point mutation in the topoisomerase 1 (Top1) gene (Fujimori *et al*., 1995; Pommier*et al*., 1999); moreover, the same mutation has been mapped in Top1 from CPT producing plants. This mutation probably enables plants to grow in the presence of CPT (Sirikantaramas *et al*., 2008) while being protected from predators. The natural CPT isomer is active only against Top1 (Hsiang *et al*., 1989; Jaxel *et al*., 1989), in fact genetically modified yeast deleted for Top1 (*Top1*Δ) was immune to CPT (Bjornsti *et al*., 1989;Eng *et al*., 1988). Two water-soluble CPT derivatives, topotecan and irinotecan have been approved by the Food and Drug Administration (FDA)

for clinical use (Figure 9) (Cuya et al., 2017). Topotecan (Hycamtin) is used to treat ovarian cancers and small-cell lung cancers (SCLC), Irinotecan (CPT-11), approved for colorectal tumors, it's a prodrug and needs to be converted to the active metabolite SN-38 by carboxylesterase (Figure 9). Hematological toxicity is a common effect due to the destruction of bone marrow progenitors. Within a day of CPT infusion, patients generally feel sick with nausea and possibly vomiting, which can generally be controlled with anti-emetic drugs. Patients may also feel tired during the first weeks of treatment. Hair loss starts 3–4 weeks after the first dose. The CPT derivatives have an intrinsic limit: the equilibrium of the α-hydroxylactone E-ring of CPTs with the carboxylate form (Figure 9), which is inactive against Top1 and tightly binds to serum albumin. For this reason, other drugs are under development, the most promising one being indolocarbazoles and the indenoisoquinolines (Pommier, 2009).

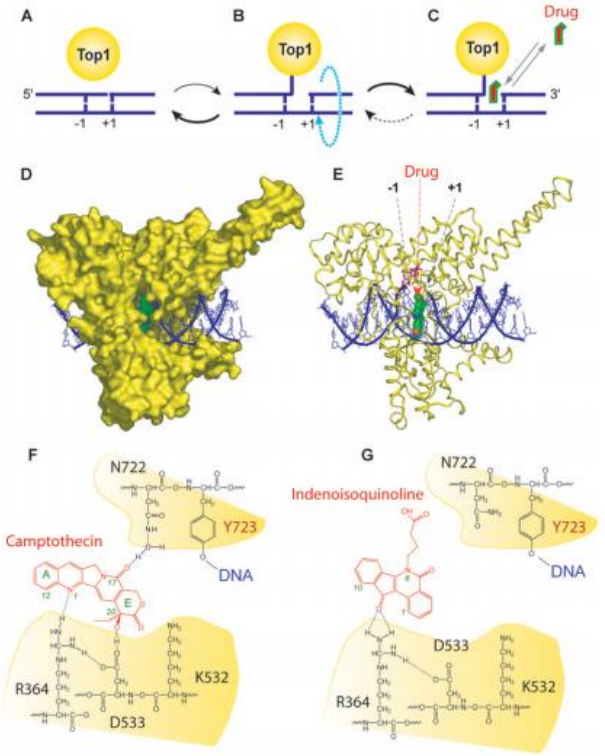


Figure 8. Interfacial inhibition by Top1 inhibitor (A) Top1 is mostly associated non-covalently with chromatin. (B) Top1 relaxes DNA by making single-strand breaks that are generated by the covalent linkage of Top1 to the 3′-end of DNA. (C) Camptothecins or non-camptothecin Top1 inhibitors bind reversibly to the Top1cc and slow down DNA religation. (D) Ternary complex including Top1 (yellow), DNA (dark blue ribbons), and an indenoisoquinoline or CPT (green and red in the middle).(E) Same structure except Top1 is in ribbon representation. (F) Hydrogen bond network between camptothecin and Top1 amino acid residues. (G). Hydrogen bond network between the indenoisoquinoline derivative MJ-238 and Top1. Note that mutation of asparagine 722 to serine (N722S), which confers resistance to camptothecin and only partially to indenoisoquinolines, is also present in camptothecin-producing plants. (Adapted from Pommier, 2009)

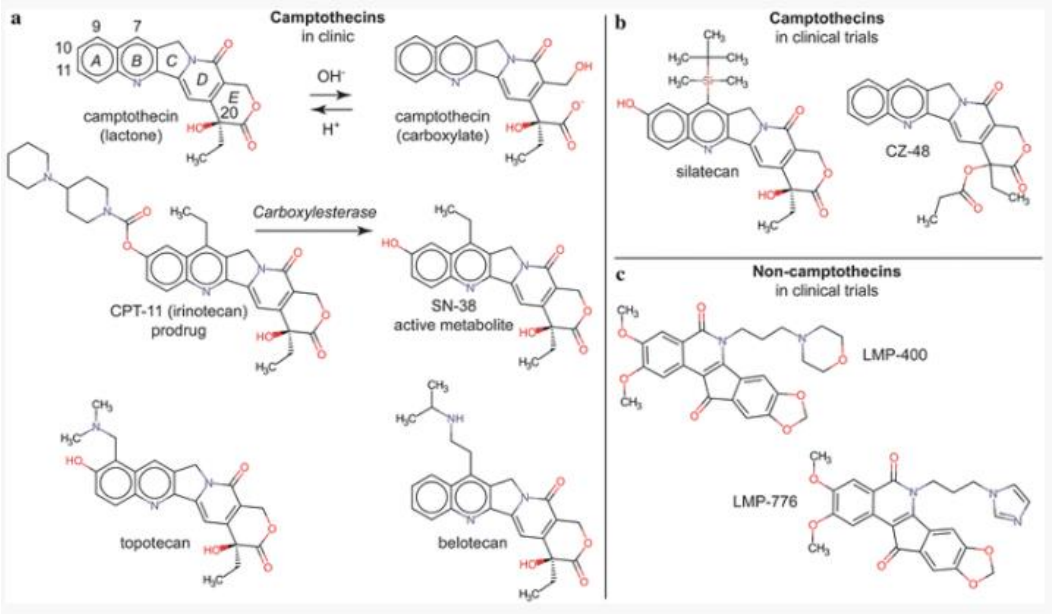


Figure 9. Structures of DNA topoisomerase IB inhibitors. Shown are the structures of camptothecin or analogs that are currently FDA approved (**a**), or in clinical trials (**b**).Non-camptothecin agents currently in clinical trials. (Adapted from Cuya et al., 2017)

1.7 Metabolites from Antarctic Organism

Organisms belonging to special ecosystem like Antarctica are a great source of natural novel products with promising bioactivities. These organisms are subjected to live in one of the most extreme and harsh environment on Earth due to low temperatures, strong winds, low nutrient and high UV radiation or combinations of these factors (Santiago et al., 2015). In order to survive in such a harsh environment these organisms require a diverse array of biochemical and physiological adaptations. Adaptation and survival of Antarctic organisms in such a stressful environment may induce the synthesis of unique metabolites having specific biological activities. Such adaptations are often accompanied by modifications in gene regulation and metabolic pathways thereby enhancing the possibility of finding new and unique functional metabolites having pharmaceutical importance.

Starting from 2001-till date a lot of new biological natural compounds with various activities have been isolated from polar organisms (Tian et al., 2017). Studies shows that metabolites derived from Antarctic organisms yield novel compounds having potent biological activities like antibacterial, antiviral, antiparasitic, antioxidant, antiproliferative, cytotoxic, enzyme inhibition etc. Interesting examples are represented by the isolation, from an extract of a *Streptomyces griseus* strain, of gephyromycin, a highly oxygenated angucyclinone exhibiting glutaminergic activity towards neuronal cells (Bringmann et al., 2005) and by palmadorin, from Antarctic nudibranch *Austrodoris kerguelenensis*, that inhibits several key enzymes involved in the JAK/STAT pathway in human erythroleukemia (Maschek et al., in 2012).

In the year 2014, Baker and co-workers isolated and characterized two new tricyclic sesquiterpenoids shagenes A&B. Exploration of the bioactivity of these two compounds revealed one to be active against the visceral leishmaniasis causing parasite, *Leishmania donovani*, with no cytotoxicity against the mammalian host. Later in the year 2016, the same group identified a new diterpene scaffold namely Darwinolide extracted from

Antarctic sponge *Dendrilla membranosa* showing a 4-fold selectivity against the biofilm phase of methicillin-resistant *Staphylococcus aureus.*

These studies demonstrate that Antarctic organism may yield an impressive array of novel compounds having potential biological activities. It is important to note that although a huge number of polar organisms have been reported very few have been deeply investigated. This situation mainly arises due to the difficulty in growing these organisms, some of which are unable to survive in normal laboratory conditions. Nowadays, advancement in the technology has led to the cultivation of organisms which were previously not accessible. New tools in the field of bioinformatics, analytics and molecular biology will help to combat limitations and allow better screening, thereby opening a new phase of research in identification of novel compounds. In the present study we screened several compounds derived from an Antarctic sponge identifying a novel inhibitor of human topoisomerase enzyme.

2. AIM OF THE STUDY

In my thesis, I have pursued two main aims:

The first one concerns the characterization of a chimeric enzyme where the N-terminal domain from *Plasmodium falciparum* has been inserted into the human enzyme counterpart. The second one concerns the investigation of the interaction of human topoisomerase 1B with novel inhibitors derived from Antarctic organism. The *Plasmodium falciparum* topoisomerase 1 (pfTop1) is an 839 amino acids monomeric enzyme encoded by a single copy gene localized on chromosome 5. The protein sequence shows a 42% identity and 50% similarity with the human homologue and structural analysis reveals that pfTop1 contains a shorter N-terminal when compared to the human topoisomerase. The N-terminal domain is the only part of the enzyme that is still not crystallized and the function of this domain is not fully known. In order to understand the importance of this domain, we have inserted the N-terminal domain of *Plasmodium falciparum* topoisomerase 1B into the human enzyme to identify the function of this domain in modulating the catalytic properties. Investigation of the structural and functional domains in chimeric enzymes may provide useful informations for the developing of selective drugs.

The second one concerns the identification of new and novel inhibitors of human topoisomerase 1B screening compounds derived from the Antarctic sponge *Artemisina plumosa*. Organisms from harsh and extreme environment are a source of diverse chemical scaffolds and novel natural products having promising bioactivities. Several studies demonstrated that Antarctic organism yielded a wide range of novel compounds having potent biological activities. The present study has identified a new natural compound derived from an Antarctic sponge having promising therapeutic properties related to cancer therapy.

3. MATERIALS AND METHODS

3.1 Media

Antibiotic: Ampicilin 100 mg/ml sterile, stored at -20ºC until use.

LB (Luria Bertoni) (1 L): 10 g tryptone, 10 g NaCl, 5 g Yeast extract, 15g Agar (only for plates) and ddH2O. YP (1L): 10 g yeast extract, 20 g bacto-peptone and ddH2O. Add 20g agar only for plates.

Synthetic complement (SC)-uracil (1L): 1.7 g Yeast nitrogen extract, 5 g Ammonium sulfate, 0.72g uracil– drop out, 2 ml NaOH 1 M, 20 g Agar (only for plates) and ddH_2O. Autoclave.

Uracil- drop-out ura- (Ade 3.4%; Trp 3.4%; His 3.4%; Arg 3.4%; Met 3.4%; Tyr 5.1%; Leu 10%; Lys 5.1%; Phe 8.5%; Thr 33.9%; Asp 16.9%).

Dextrose/raffinose/galactose (stock 20%): 20 g in 100 ml ddH2O. Sterile filtered (membrane 0.22 μm). Add 2% of sugar into the yeast media.

3.2 Chemicals, yeast strains and plasmids

Anti-FLAG M2 monoclonal affinity gel, FLAG peptide and Anti-FLAG M2 monoclonal antibodies were purchased from Sigma-Aldrich. In order to express the hTop1and hTop1 (pf-N-term) enzymes we used saccharomyces cerevisiae Top1 null strain EKY3 (ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1::TRP1, MATα) and MBY3 (MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, rad52Δ::LEU2). Single copy plasmid YCpGAL-e-hTop1 was used to express the three enzymes under galactose inducible promoter. The N-terminal sequence of the epitope-tagged construct YCp-GAL-e-hTop1 contains FLAG: DYKDDDY indicated as ‘e’, which is recognized by the M2 monoclonal antibody. To generate hTop1(pf-N-term) only N-terminal (1-153) flagged pfTop1 was constructed by using Pyes2.1 TAG pfTop1 as template and two set of primers 5´-ATGGACTACAAGGACGAC-3´ and 5´-A TCGGTTTGATCATCAATCTT-3´. In the next step, a fragment containing core domain (215-635), linker domain (636-712) and C-terminal domain

(713-765) of hTop1 was generated using by Ycptag hTop1 as template and two sets of primers were 5′-AAGATTGATGATCAAACCGATATCAAGTGGAAATTCCTAGA ACATAAAGGT-3´and 5´-GGCTGTCAGTTCTTTTAGCTGCTGCTGTAGCGTGATGG AGGCATTGTATGT-3´. The Products were obtained using a Taq DNA polymerase (Sigma- Aldrich) and the specific band was purified from 0.7% agarose gel in 1X TBE buffer (48 mM Tris, 45.5 mM boric acid, 1 mMEDTA) using the gel extraction kit QIAEX II purchased by Qiagen. The hTop1 and hTop1 (pf-N-term) constructed was cloned into pYES2.1/V5-His-TOPO expression vector (Invitrogen), according to the provided manufacturer protocol. The cloning reaction was transformed into XL10-Gold E. coli cells (Agilent Technologies), and a positive clone was identified by sequencing the extracted plasmid DNA.

3.3 Protein purification

In order to purify hTop1 and hTop1 (pf-N-term) they were cloned in a single copy plasmid, YCpGAL1 and in pYES2.1/V5-His-TOPO respectively, under a galactose inducible promoter. By using lithium acetate procedure hTop1and hTop1 (pf-N-term) were transformed in top1 null EKY3. The cells were then grown overnight on SC-Uracil (synthetic complete medium lacking uracil) with 2% dextrose. In next step, they were diluted 1:100 in SC-uracil plus 2% raffinose, at OD600 = 1.0, the cells were induced with 2% (v/v) galactose for 6h. Cells were then harvested by centrifugation, followed by washing with cold water and resuspended in 2ml buffer per gram of cells using a buffer containing 50mM Tris/HCL, pH 7.4,1 mMEDTA, 1mM EGTA,10% (v/v) glycerol, protease inhibitor cocktail and supplemented with 0.1mg/ml sodium bisulfite and 0.8 mg/ml sodium fluoride. 0.5 volume of glass beads having the diameter of 425-600 mm was added in order to disrupt the cells by vortexingfor 30 seconds altering with 30 seconds on ice. After vortexing, they were centrifuge at 12000 rpm for 30 minutes to collect supernatant. The collected supernatant was then applied to an ANTI-FLAG M2 Affinity Gel column equilibrated as described by the manufacturer (sigma) to obtain homogenous protein. The column was

washed with 20 column volumes of TBS (50mM Tris/HCL and 150mM KCl, pH 7.4). Elution of hTop1 and hTop1(pf-N-term) was performed by adding five column volumes of a solution containing 100 µg/ml FLAG peptide (Tris-buffered saline). Fractions of 500µl were collected and a final concentration of 40% (v/v) glycerol was added; all fractions preparations were stored at -20ºC. The eluted purified fractions were resolved by SDS/PAGE protein concentration and integrity was measured through immunoblot assay using the epitope-specific monoclonal antibody M2. The *in-vitro* experiments have been performed using three units of purified hTop1 and hTop1 (pf-N-term).

3.4 Unit determination

One unit of an enzyme is defined as the amount of enzyme that catalyzes the relaxation of 0.5µg of negatively supercoiled DNA in 15 minutes at 37o in a total reaction volume of 30µl.

3.5 DNA relaxation assays

The activity of hTop1 hTop1(pf-N-term) was evaluated by decreasing mobility of the relaxed isomers of supercoiled pBlueScript KSII (+) DNA in an agarose gel. The relaxation activity of hTop1and hTop1 (pf-N-term) (3 units) was assayed in 30µl of reaction volume containing 0.5µg of negatively supercoiled pBlue-script KSII (+) DNA present in both dimeric and monomeric form containing reaction buffer (20 mM Tris–HCl pH 7.5, 0.1 mM Na2EDTA, 10 mM MgCl2, 50µg/ml acetylated bovine serum albumin and 150 mM KCl). The reactions were stopped using 0.5% SDS at each time-course point at 37°C. The samples were resolved at 1% agarose gel running buffer containing 48 mM Tris, 45.5 mM boric acid, 1 mM EDTA. DNA was visualized by staining of the gel with 0.5µg/ml ethidium bromide and destained with water and gel image was photographed using a UV transilluminator.

3.6 Cleavage kinetics using CL14/CP25 oligonucleotide substrate

For analyzing cleavage kinetics, an oligonucleotide suicide substrate namely CL14 (5'-GAAAAAAGACTTAG-3') was used. This oligonucleotide substrate was radiolabeled with [γ-32P] ATP at its 5′ end. The complementary strand of this oligonucleotide namely CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3') was phosphorylated with unlabeled ATP at its 5′ end. The suicide cleavage reactions were carried out by incubating 20nM of the duplex with 3 units of hTop1 and hTop1(pf-N-term) in 10 mM Tris pH 7.5, 5 mM MgCl2, 5 mM CaCl2 and 150 mM KCl at 25°C in a final volume of 60µl.Aliquots of 5µl were removed at different time intervals and for stopping the reaction 0.5% (w/v) SDS was added. The samples were then subjected to ethanol precipitation. After this the samples were digested by adding 5µl of 1mg/ml of trypsin and incubated at 37ºC for 60 minutes. The samples were evaluated by denatured polyacrylamide gel electrophoresis (7M urea/20%) in TBE running buffer (48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA).

3.7 Cleavage kinetics using CL14-U/CP25 oligonucleotide substrate

Oligonucleotide CL14-U (5′-GAAAAAAGACTUAG-3′) was radiolabelled with [γ-32P] ATP at its 5′-end and annealed with CP25 to obtain the CL14-U/CP25 suicide substrate. The hTop1and hTop1(pf-N-term) of 3 units were incubated with 20nM suicide substrate in 20 mM Tris–HCl pH 7.5, 0.1 mM Na_2EDTA, 10 mM MgCl2, 5 µg/mL acetylated BSA, and 150 mM KCl. 5µl of aliquots were removed at different time points and for stopping the reaction 0.5% (w/v) SDS was used. The samples were directly loaded without ethanol precipitation and trypsin digestion. The samples were analyzed by denatured polyacrylamide gel electrophoresis (7M urea/20%) in TBE running buffer (48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA). In both the experiments the percentage of cleaved substrate (Cl1) was determined by Phosphor Imager and Image Quant software and normalized on the total amount of radioactivity in each lane.

3.8 Religation kinetics

Analyses of religation kinetics was carried out using oligonucleotide substrate CL14 (5′-GAAAAAAGACTTAG-3′) which was radiolabelled with [γ-32P] ATP at its 5′-end. The complementary strand of this oligonucleotide namely CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3') was phosphorylated with unlabeled ATP at its 5′ end. The CL14 strand of the oligonucleotide was annealed with a 2-fold molar excess of CP25 to obtain partially duplex CL14/CP25 suicide substrate. This radiolabeled oligonucleotide (CL14/CP25) was incubated with 3 units of hTop1and hTop1(pf-N-term) for 60 min at 25 °C and then for additional 30 min at 37 °C in 20 mM Tris– HCl pH 7.5, 0.1 mM Na2EDTA, 10 mM MgCl2, 50 µg/ml acetylated BSA and 150 mM KCl. After the formation of the cleavage complex (Cl1), 5 µl aliquot was removed representing time 0. In the next step, 200-fold molar excess of R11 oligonucleotide (5′-AGAAAAATTTT-3′) over duplex CL14/CP25 was added to initiate relegation kinetics. Aliquots containing 5µl was removed at different time points and the reaction was stopped by addition of 0.5% SDS. The samples were then subjected to ethanol precipitation. After this the samples were digested by adding 5µl of 1mg/ml of trypsin and incubated at 37ºC for 60 minutes. Since trypsin doesn't digest hTop1B completely therefore there is a trypsin resistant peptide which remains attached to the substrate causing the 12nt (Cl1) oligo to run slower than the uncleaved band in the gel. The samples were then analyzed by using 7 M urea/20% denaturing polyacrylamide gelelectrophoresis running buffer containing 48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA.The religation percentage was quantified by Image Quant software, and then the ratio between the religation band and the total radioactivityfor each lane was plotted as the time function and normalized to the plateau value.

3.9 Cell viability

For this experiment, we used isogenic yeast strains MBY3 (*top1Δ*, *rad52 Δ*) which were transformed with *URA3* marked vector and plated onto selective SC-uracil plates supplemented with 2% dextrose. The cells were then grown overnight on SC-uracil with 2% dextrose. At an optical density of A600=1.0, the cells were diluted 1:100 in SC-uracil containing 2% raffinose. After this the cells were again diluted to an optical density 0.3 in SC-uracil medium with 2% raffinose and incubated for further 1 hour. Following this incubation, cell was removed for representing time 0 and serially diluted to be plated on SC-uracil medium with 2% dextrose. The cells were then induced for expression by using 2% galactose. At different time intervals the cells were removed and serially diluted before plating on the respective selective media. The plates were then incubated for 3 days at 30ºC and counted for viable cells. The graph was plotted as the number of viable cells obtained at time 0 against time.

3.10 Cell Culture

The PC3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was authenticated by the purchaser cell bank. Cells were grown as monolayer in RPMI-1640 Medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) and cell cultures were maintained at 37°C, in a humidified atmosphere of 5% CO_2. Cells were passaged after being detached from culture flasks with 0.05% trypsin and 0.002% EDTA solution. All media and supplements for cell cultures were obtained from Hyclone (Logan, UT, USA).

3.11 Treatment and Primary analyses

For treatments, cells were seeded at densities ranging from $4x10^4/cm^2$ and $6x10^4/cm^2$ and maintained in culture for 24h before the addition of FrH1 and FrH2. Stock solutions (5.26mM) of FrH1 and FrH2 were prepared in absolute DMSO. Cells were treated with increasing doses of extract fractions

(ranging from 20 to 200µM, max final concentration DMSO: 3.7%) and maintained in culture 24h before to be the analyzed.

The cytotoxicity of H1 was evaluated by Trypan blue dye exclusion method. The effects of H1 treatments have been compared with those of untreated cells and of control performed with DMSO (final concentration present in the higher dose of H1)

4. RESULTS

4.1 Construction of Chimeric Enzyme namely hTop1 (pf-N-terminal)

To generate hTop1 (pf-N-terminal), three different sets of PCR reaction were carried out. In the first step, only the N-terminal (1-153) flagged pfTop1 was constructed by using Pyes2.1 TAG pfTop1 as template and two set of primers 5´- ATGGACTACAAGGACGAC-3´ and 5´- ATCGGTTTGATCATCAATCTT-3´. In the next step, a fragment containing core domain, linker domain and C-terminal domain of hTop1 was generated using Ycp tag hTop1, using two sets of primers 5´- AAGATTGATGATCAAACCGATATCAAGTGGAAAT TCCTAGAACATAAAGGT-3´and 5´- GGCTGTCAGTTCTTTTAGCTGCTGCTGTAGCGTGATGGAGGCATT GT ATGT-3´ were used. Finally, the last set of primers 5´- CAGCTAAAAGAACTGACA GCC-3´ and 5´- CTAAAACTCATAGTCTTCATCAGCCATGTC-3´ was used to generate the plasmid chimera. The three separate sets of reactions were ligated to obtain the full-length domain-swapped product, hTop1 (pf-N-terminal), comprising *P. falciparum* N-terminal domain embedded in hTop1 core, linker and C-terminal domains (Figure 10). The cloning reaction was transformed into XL10-Gold *E. coli* cells and positive clones were identified by sequencing the extracted plasmid DNA. hTop1 (pf-N-terminal) were cloned in a single copy plasmid under a galactose-inducible promoter. Proteins were purified through affinity chromatography as described in material and methods. After purification, we determined the unit of the enzyme as the amount of enzyme that catalyzes the relaxation of 0.5µg of negatively supercoiled pBlue-Script DNA in 15 minutes at 37°C in a total reaction volume of 30µl. The *in-vitro* experiments have been performed using three units of purified hTop1 (pf-N-terminal) comparable to the same units of hTop1.

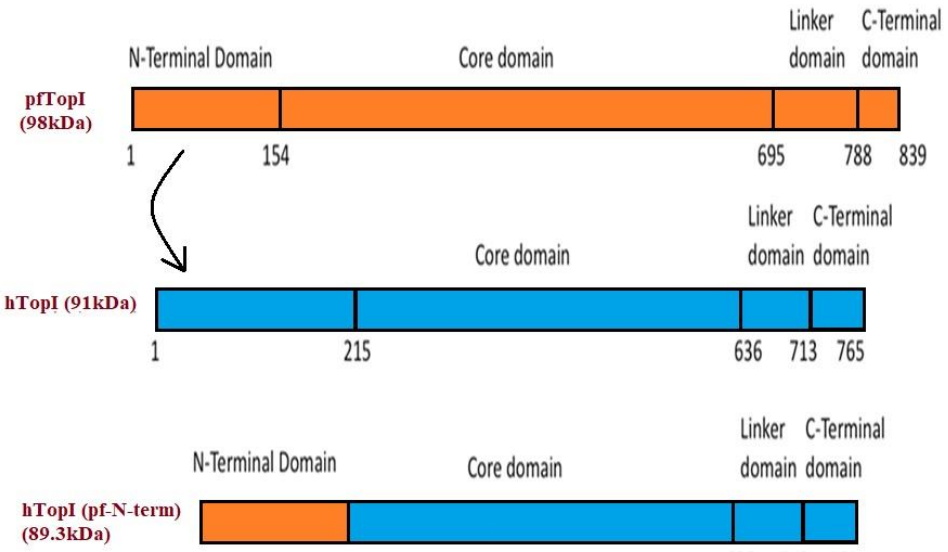


Figure 10. Schematic diagram of generation of human/plasmodial hybrid enzyme namely hTop1 (pf-N-terminal)

4.2 Relaxation assay

The relaxation activity of hTop1 and hTop1 (pf-N-terminal) enzymes, has been assessed by incubating 3 units of both enzyme with 0.5µg of a negatively supercoiled plasmid at 37°C in a time course experiment from 0.5 to 60 minutes. The products have been resolved by agarose gel electrophoresis. As shown in Figure 11, the complete relaxation of supercoiled DNA is observed in hTop1 enzyme after 0.5 minutes (Figure 11, lane 1) while the hTop1(pf-N-term) relaxed the DNA after 4 minutes (Figure 11, lanes12). This result indicates that the chimeric enzyme has slower relaxation kinetics when compared to the wild type protein.

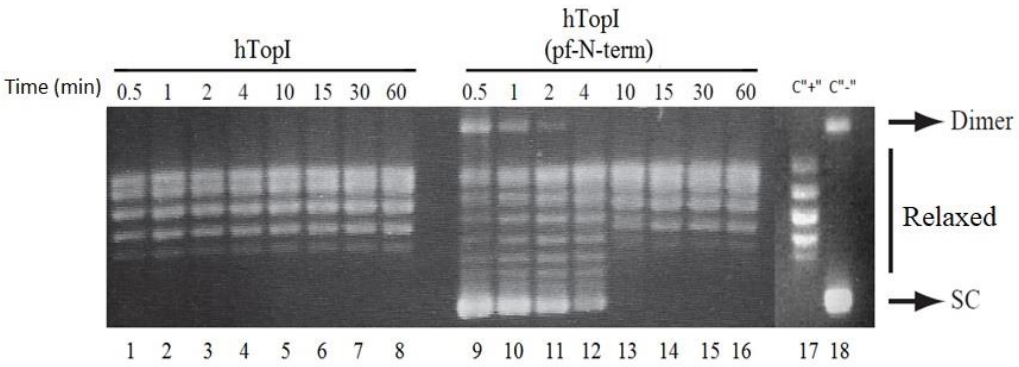
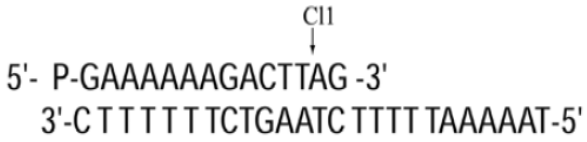


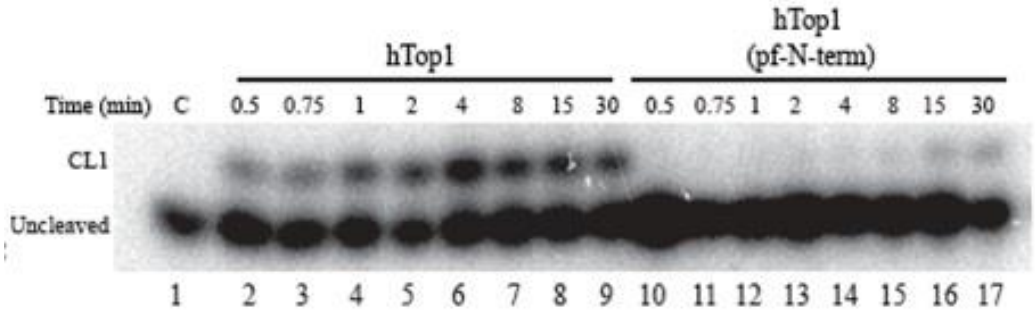
Figure 11 . Relaxation of the negatively supercoiled plasmid in a time course experiment in presence of hTop1 (lane 1-8), hTop1 (pf-N-term) (lane 9-16). In lane 17 an active purified fraction of topoisomerase is added whereas in lane 18 no protein is added.

4.3 Cleavage with CL14/CP25 suicide substrate

The cleavage activity of the hTop1 and hTop1 (pf-N-term) has been compared in a time course experiment using a CL14/CP25 suicide cleavage substrate containing the preferential hTop1 cleavage site, which is indicated by an arrow in the top panel of Figure 12. In detail, a 5' end radiolabelled oligonucleotide CL14 (5'-GAAAAAAGACTTAG-3') has been annealed to the CP25 (5'-TAAAAATTTTTCTAA GTCTTTTTTC-3') complementary strand, to produce a duplex with an 11 base 5' single-strand overhang (Figure 12). In this experiment the religation step is avoided because the AG-3' dinucleotide is too short to be religated, leaving the enzyme covalently attached to the 12 oligonucleotide 3'-end. 20nM of suicide substrate has been incubated with 3 units of wild type and hTop1(pf-N-term) enzyme in a time course experiment. The reactions were stopped at increasing time points from 0.5 to 30 minutes. After ethanol precipitation and trypsin digestion, the products have been resolved in a denaturing urea polyacrylamide gel (Figure 12A). As evident from figure 12A the cleavage rate is very slow or almost absent for hTop1(pf-N-term) when compared to hTop1. Percentage of the cleaved fragment (CL1), normalized to the total radioactivity amount in each lane, has been plotted against time for the hTop1(pf-N-term) (Figure 12B, triangle), and the hTopI enzyme (Figure 12B square) confirming the lower cleavage kinetics of the chimera.


Cl1
5'- P-GAAAAAAGACTTAG -3'
3'-C T T T T T TCTGAATC TTTT TAAAAAT-5'

A)


hTop1
hTop1
(pf-N-term)
Time (min) C 0.5 0.75 1 2 4 8 15 30 0.5 0.75 1 2 4 8 15 30
CL1
Uncleaved
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

B)

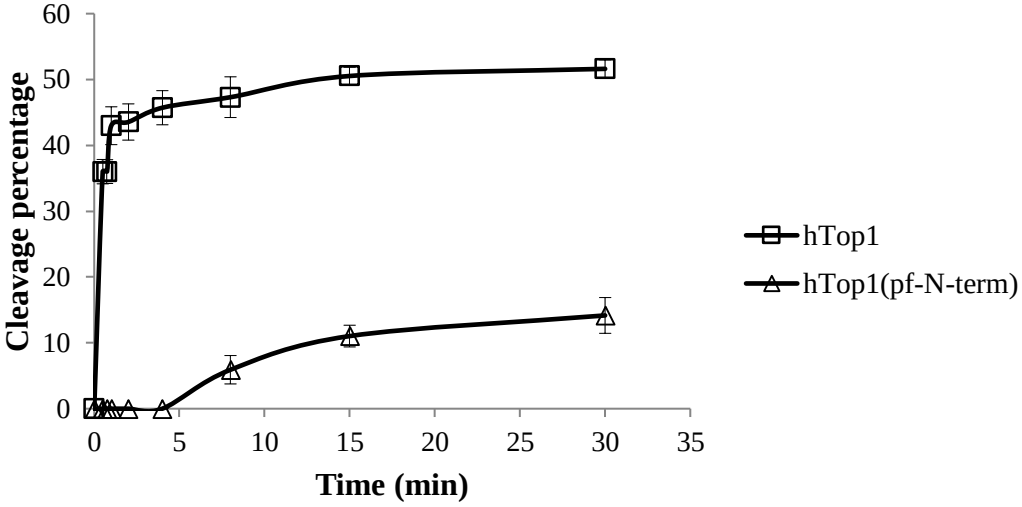
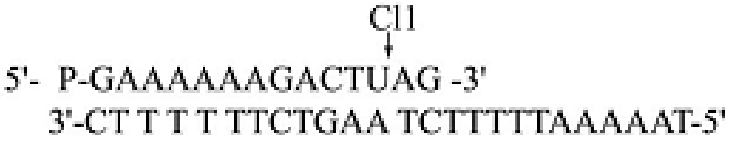


Figure 12. Cleavage kinetics using suicide substrate. (A) Time course (0.5-30 minutes) of the cleavage reaction of the purified hTop1 (lanes 2-9) and hTop1 (pf-N-term) (lane 10-17) with the CL14/CP25 suicide substrate shown at the top of the Figure, lane 1 no protein added. The arrow indicates the preferred cleavage (CL1) site for the enzyme. (B) Percentage of cleaved suicide substrate plotted against time for hTop1 (square), hTop1 (pf-N-term) (triangle). Three independent experiments were performed to obtain an average value and the standard deviation for error bar.

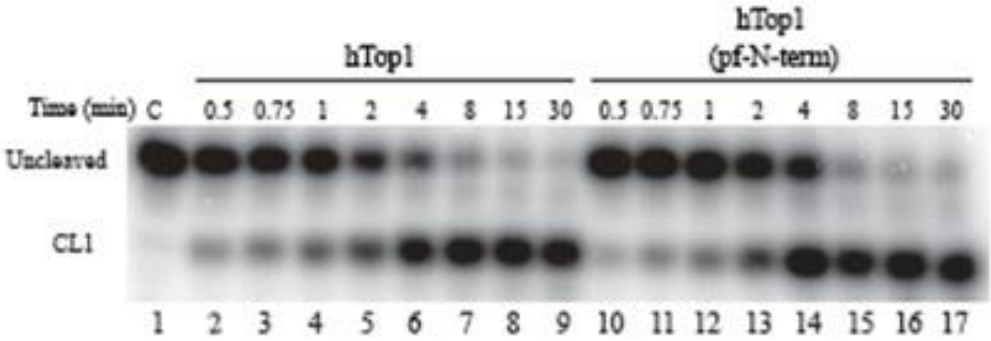
4.4 Cleavage with CL14-U/CP25 suicide substrate

In order to better evaluate the cleavage rate between the two enzymes a different cleavage substrate has been used to follow the rate of cleavage. CL14-U (5'-GAAAAAAGACTUAG-3') oligonucleotide, containing an uracil at the preferred hTop1 cleavage site, has been used to perform a different cleavage experiment. This oligonucleotide, having the deoxyribose-thymine (dT) in position 12 substituted by a ribo-Uracil (rU), has been annealed to the CP25 (5'-TAAAAATTTTTCTAAGTCTTTTTTC-3') complementary strand, to produce a duplex with an 11-base 5'- single-strand extension. With this oligonucleotide the enzyme does not remain trapped over the substrate since, after the cutting at the preferential site, the 2'-OH of the ribose attacks the 3'- phosphotyrosyl linkage between the enzyme and the ribonucleotide, releasing the enzyme and leaving a 2',3'-cyclic phosphate end (Figure13A). Three units of both enzyme were incubated with 20nM of radiolabelled ribo modified CL14-U/CP-25 substrate and reaction was stopped after different time intervals from 0.5 minutes to 30 minutes. As evident from figure 13B hTop1(pf-N-term) enzyme shows the same cleavage rate as hTop1 with this substrate (Figure 13B, compare lane 10-17 with lane 2-9). The clevage percentage has been evaluated by plotting the cleaved products, normalized to the total amount of radioactivity in each lane, as function of time (Figure 13C).

A)


Cl1
5'- P-GAAAAAAGACTUAG -3'
3'-CT T T T TTCTGAA TCTTTTTAAAAAT-5'

B)


hTop1
hTop1
(pf-N-term)
Time (min) C 0.5 0.75 1 2 4 8 15 30 0.5 0.75 1 2 4 8 15 30
Uncleaved
CL1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

C)

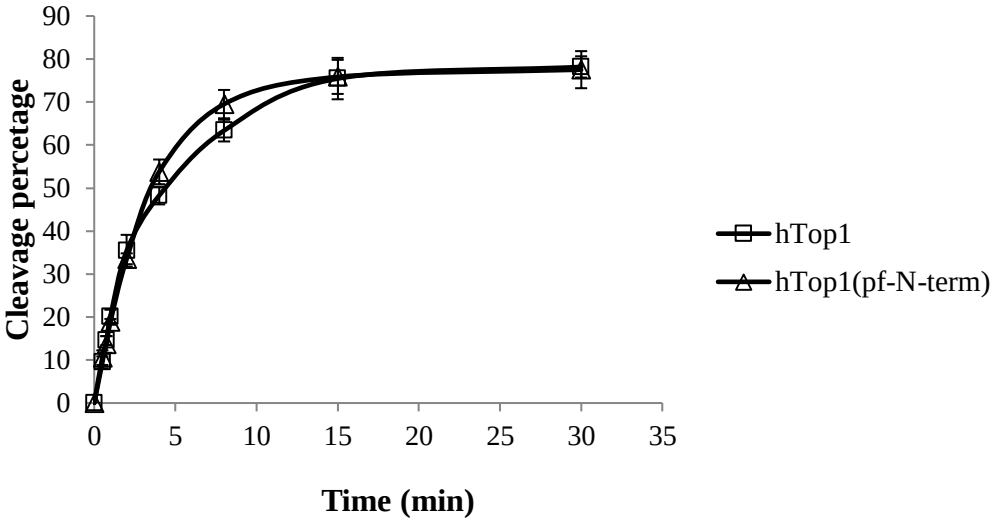
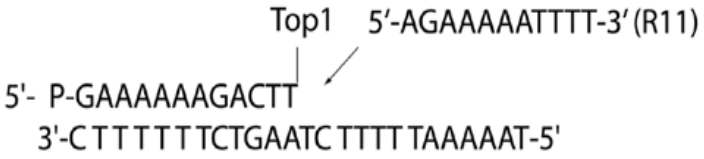


Figure 13. (A) Sequence of the CL14-U/CP25 substrate. The hTop1 cleavage site containing a scissile ribonucleoside monophosphate is indicated by an arrow. (B) Time course (0.5-30 minutes) of the cleavage reaction of purified hTop1 (lanes 2-9), hTop1 (pf-N-term) (lanes 10-17) with the CL14-U/CP25 substrate. Lane 1 no protein added. CL1 represents the DNA substrate cleaved by the enzymes at the preferred cleavage site. (C)Percentage of cleaved substrate plotted against time for the reaction with hTop1 (square), hTop1 (pf-N-term) (triangle). Data shown are means ± SD from 3 independent experiments.

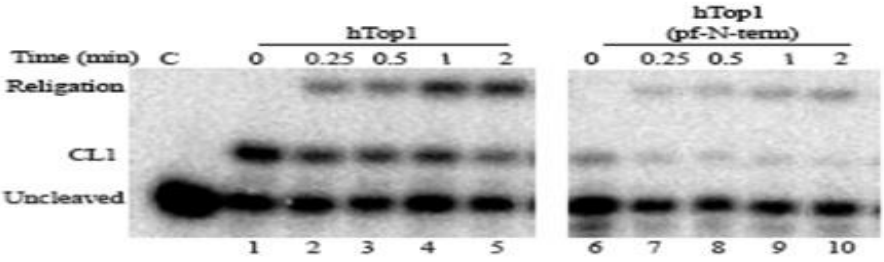
4.5 Religation Kinetics

Religation kinetics of hTop1 and hTop1 (pf-N-term) have been assessed by incubating 3 units of each enzymes with 20nM suicide substrate CL14/CP25 to generate the claveable complex. After the formation of the cleavage complex, a 200-fold molar excess of complementary R11 oligonucleotide (5'-AGAAAAATTTT-3') has been added (Figure 14A). Aliquots have been removed at different times from 0.25 min to 2 min and stopped by adding 2.5% SDS. The reaction products have been analyzed by denaturing PAGE (Figure 14B) and plotted as function of time in figure 14C. The result indicates that the wild type efficiently religates the substrate (Figure 14B lane 2-5), whereas in the case of the hTop1 (pf-N-term) the amount of the religation products over time is very low or almost absent (Figure 14B lane 7-10). The percentage of religation has been plotted as function of time in Figure 14C. This result indicates that the substitution of the N-terminal strongly perturbs the religation property of the enzyme.

A)


Top1
5′-AGAAAAATTTT-3′ (R11)
5'- P-GAAAAAAGACTT
3'-C T T T T T TCTGAATC TTTT TAAAAAT-5'

B)


hTop1
hTop1
(pf-N-term)
Time (min) C
0 0.25 0.5 1 2
0 0.25 0.5 1 2
Religation
CL1
Uncleaved
1 2 3 4 5
6 7 8 9 10

C)

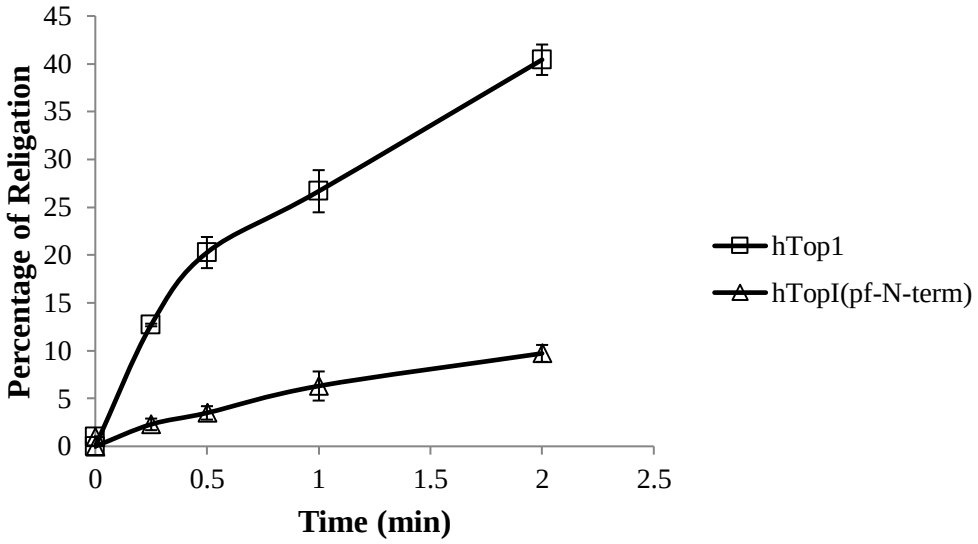


Figure 14. (A) Religation kinetics. The R11 complementary oligonucleotide (shown at the top of the figure) is added to the suicide cleavage complex (CL1) to the hTop1(lane 2-5) or hTop1(pf-N-term) (lane 7-10); lanes 1 and 6 represent the time 0 for hTop1 and hTop1 (pf-N-term) mediated reactions, respectively. "CL1" represents the DNA fragment cleaved at the preferred enzyme site; "religation" is the restored fully duplex oligonucleotide representing the final product of the religation reaction. (B) Plot of the religation percentage as function of the time for the hTopI (square) and hTop1 (pf-N-term) (triangle) respectively. Data shown are means ± SD from 3 independent experiments.

4.6 Cell viability assay in a rad52Δ yeast strain

To evaluate the in vivo consequence of hTop1 and hTop1 (pf-N-term) expression and viability, we have performed a cell viability assay in liquid culture in a DNA double strand break repair deficient strain (MBY3-rad52Δ). MBY3 cells transformed with a single copy plasmid (YCp) expressing the hTop1 (pf-N-term), hTop1 and the vector have been induced with galactose. At various time points, aliquots have been removed and plated onto selective medium to count the viable cells. Expression of an active hTop1 enzyme into a topoisomerase deficient yeast strain (top1Δ) defective in double-strand breaks repair (rad52Δ) causes cell damage and decrease in cell viability as shown in Figure 15 (square line). In case of hTop1 (pf-N-term) figure 15 (triangle line) shows a decrease in the number of viable cells suggesting a lower DNA damage. This result indicates that the chimeric protein is less active or binds the DNA at lower level or it has different substrate specificity. Work is in progress to understand which one of the three hypotheses is the most reliable one.

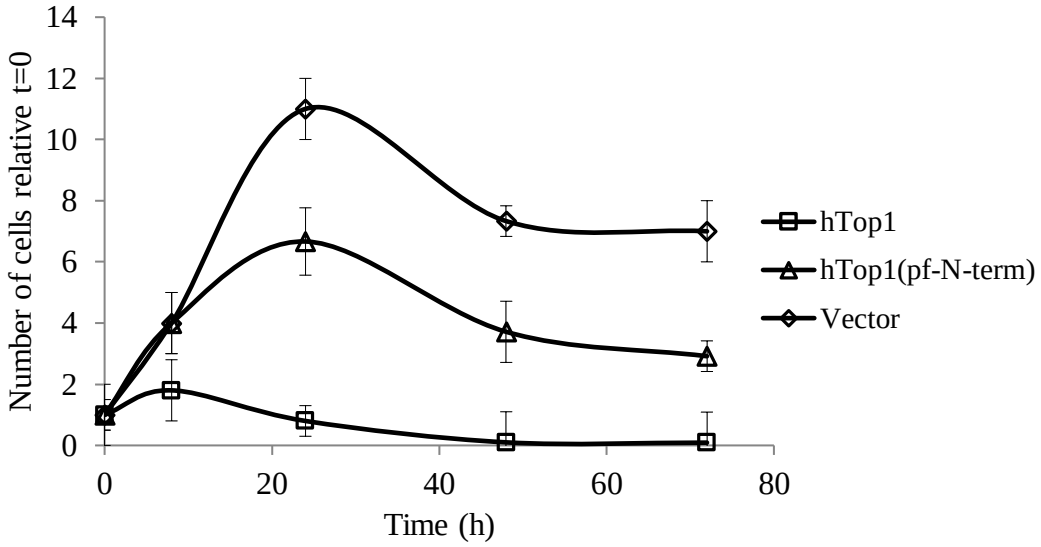


Figure 15. MBY3 (rad52) strains were transformed with the galactose-inducible GAL1 promoter. Exponentially growing cultures of individual transformants induced with galactose expressing the respective protein were serially 10-fold diluted, and 5µl aliquots were spotted onto SC-uracil plates supplemented with 25 mM HEPES (pH 7.2), 2% dextrose. The number of cells forming was assed at 30ºC and plotted relative to the number obtained at t=0, in case of hTop1 (square) and hTop1 (pf-N-term) (triangle) respectively. Data shown are average values ±SD from 3 independent experiments.

4.7 Investigating interaction of hTop1 with inhibitors derived from Antarctic invertebrates

The extracts from Antarctic invertebrates were obtained by Prof. Baker and co worker from University of South Florida, USA. Every year he and his group do several expeditions to Antarctica to collect Antarctic organisms. After returning to USA, they prepare the extracts by precipitation using solvents of different polarity, this result in the elution of several fractions per organism. These fractions were sent to us for primary screening. For the ease of understanding this part of my thesis is divided in to two parts:

a) Screening of extracts derived from Antarctic invertebrates
b) Identification of the metabolite responsible for inhibition

a) Screening of extracts derived from Antarctic invertebrates

In the first phase of the screening, a total of 750 extracts were screened. In detail, supercoiled plasmid DNA (0.5µg/µl) was incubated with 0.16µg/µl of each extract for 5 minutes at 37°C. Following this incubation 1µl of purified fraction of hTop1 was added to the mix and incubated for 60 minutes at 37°C. The reactions were then stopped by adding 0.5% of SDS and products were resolved by agarose gel electrophoresis. Primary screening resulted in the identification of 94 extracts which were found positive for hTop1 inhibition. We observed two patterns of inhibition i.e. reversible and irreversible. Out of 94 inhibitions 89 were irreversible and only 5 were found to be reversible inhibitor of hTop1 (Table 4). Our main interest was focused on characterization of metabolites responsible for reversible inhibition.

Table 4: Data showing total number of extracts screened and inhibition obtained

TOTAL EXTRACTS	**750**
TOTAL INHIBITION	94
IRREVERSIBLE INHIBITION	89
REVERSIBLE INHIBITION	05

b) Identification of the metabolite responsible for inhibition

In the second phase of screening 1 out of 5 extracts was selected for further purification. This extract comes from Antarctic sponge known as *Artimisina plumosa.* In the second phase, Prof. Baker and group used MPLC to purify the extract and eluted 18 fractions. These 18 fractions were again sent to us for further screening to identify the metabolite responsible for inhibition. In detail, a negatively supercoiled plasmid DNA (0.5µg/µl) was incubated with 200 µM (final concentration) of each fraction for 5 minutes at 37°C. Following this incubation 1µl of purified fraction of hTop1 was added to the mix and incubated for 60 minutes at 37°C. The reactions were then stopped by adding 0.5% of SDS and products resolved by agarose gel electrophoresis. I identified 9 fractions (Figure 16) out of 18 which were responsible for the inhibition.

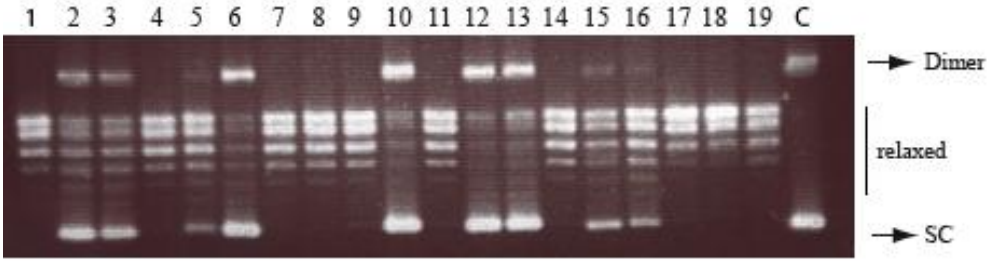
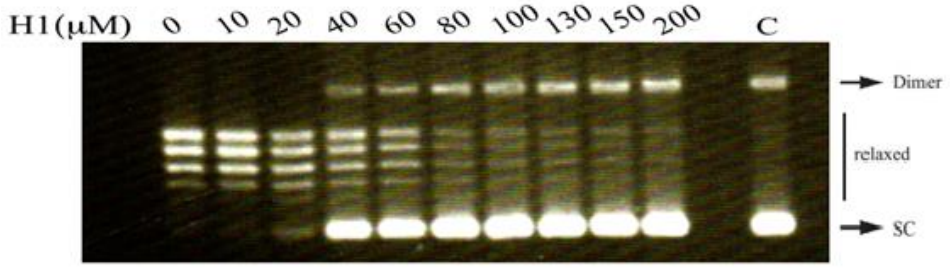


Figure 16. Relaxation of supercoiled plasmid in presence of hTop1 (lane 1-19) and of the different MPLC fractions from *Artemisina Plumosa* extract (lane 2-19), lane1: no fraction added; C: no protein added. The reaction products are resolved in an agarose gel and visualize after staining in ethidium bromide. The two forms of plasmid DNA are indicated as Dimer and supercoiled (SC).

Next, I performed dose dependent experiment to determine the minimum dose concentration for the extract to inhibit hTop1. For this purpose, I incubated negatively supercoiled DNA with different concentration of partially purified fraction (10µM, 20µM, 40µM, 60µM, 80µM, 100µM, 130µM, 150µM, 200µM) for 5 minutes at 37°C. After this incubation 1µl of purified fraction of hTop1 to the mix and incubated for 60 minutes at 37°C. The reactions were stopped with 0.5% of SDS and products were resolved using agarose gel electrophoresis. Based on the dose dependent experiment the most concentrated fraction namely H1 was selected i.e. the one inhibiting at the lowest concentration (Figure 17A). Time course experiments were carried out to check for its pattern of inhibition which in our case should be reversible. For performing time course experiments, I incubated 0.5µg negative supercoiled plasmid with the fraction for 5 minute at 37°C. After the incubation, 1µl of purified fraction of hTop1 was added to the mix. I stopped the reaction at different time points ranging from 0.5 to 60 minutes. The samples were resolved using agarose gel electrophoresis. The results indicate that this fraction inhibits hTop1 reversibly at a concentration of 30µM (Figure 17B).

A)



B)

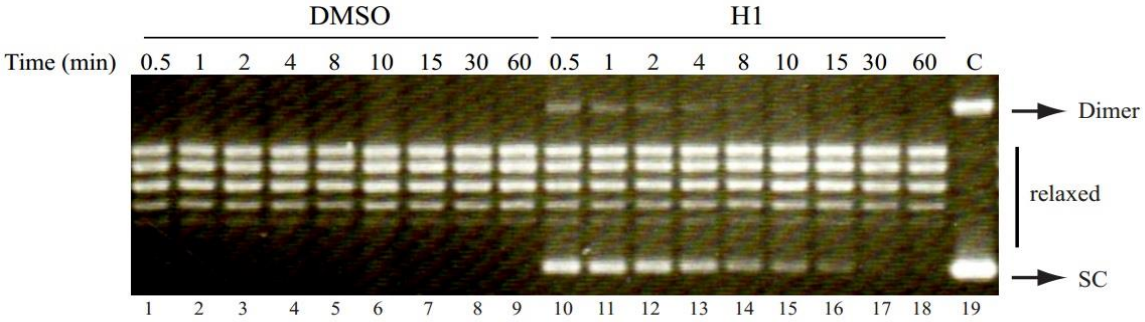
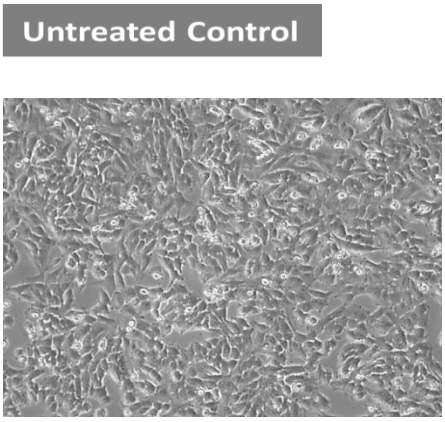


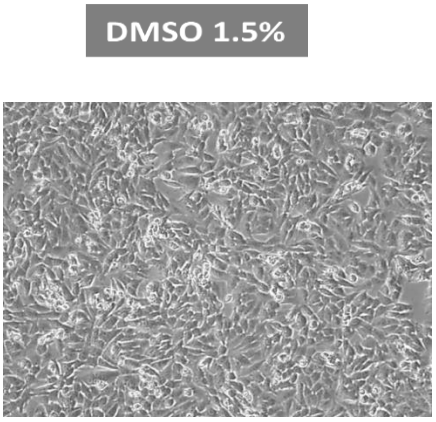
Figure 17. A) Dose dependent effect of metabolite H1 on the relaxation activity of hTop1 enzyme B)Relaxation of the negatively supercoiled plasmid by hTop1 in a time course experiment in presence of DMSO (lane 1-9) and H1 metabolite derived from Antarctic sponge *Artimisina plumosa)* (lane 10-18). Lane 19 no protein is added

We also tested inhibition activity of this partially purified fraction in tumour cell line; this work was carried out in collaboration with Dr. Annalucia Serafino from National Research Council, Rome, Italy. Preliminary experiments were conducted for assessing the antiproliferative efficacy of the fraction that exhibited inhibitory activity against hTop1. For this purpose PC3 prostate adenocarcinoma cells purchased from the American Type Culture Collection (ATCC), have been cultured and maintained as described in the Materials and Methods. . Thereafter, cells were treated with increasing doses of fraction (ranging from 10 to 200µM) and maintained in culture 24-48h before the analysis. The cytotoxicity of extract fraction was evaluated by Trypan blue dye exclusion method. Results indicate that a higher concentration of 80µM the metabolite was able to affect cell viability at a higher extent compared to untreated and DMSO treated controls as demonstrated by phase contrast microscopy (Figure 18) and by cell viability assay (Figure 19). Here, we would also like to mention that these results are not conclusive as the effect can also be due to the higher percentage of DMSO and further validation with crude compound derived from this extract is necessary to produce conclusive data.

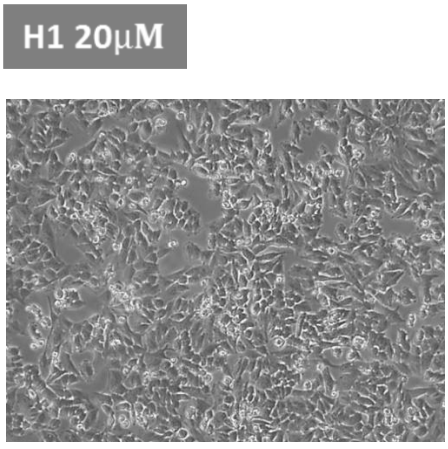
A



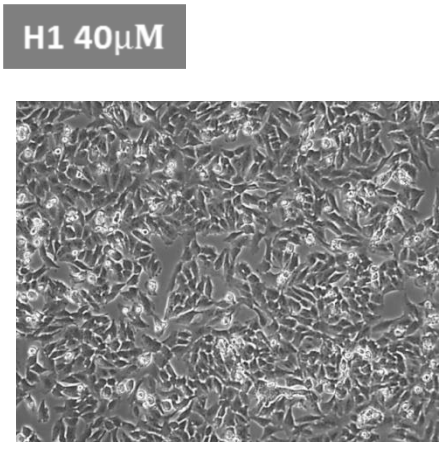
B



C



D



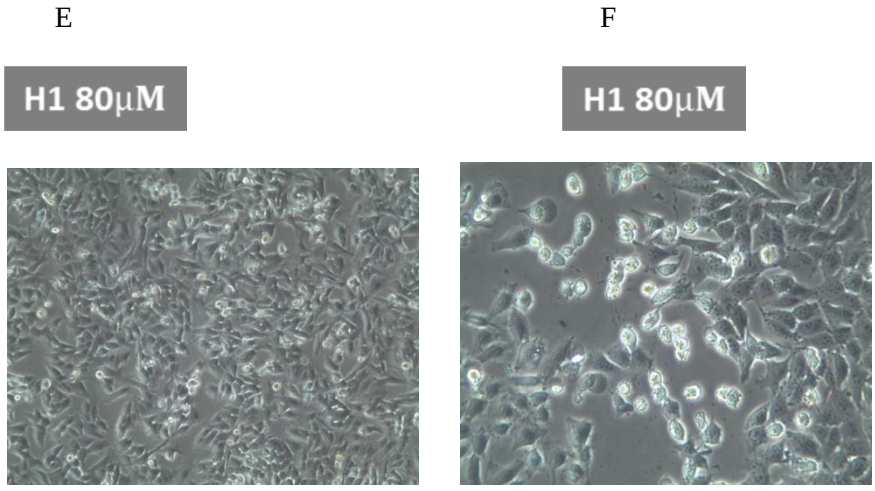


Figure 18. Phase contrast microscopy of PC3 cell line A) Untreated B) cells exposed to 1.5% DMSO C) Cells exposed to 20μM extract fraction D) cells exposed to 40μM extract fraction E) cells exposed to 80μM extract fraction F) Magnification of cells exposed to 80μM extract fraction

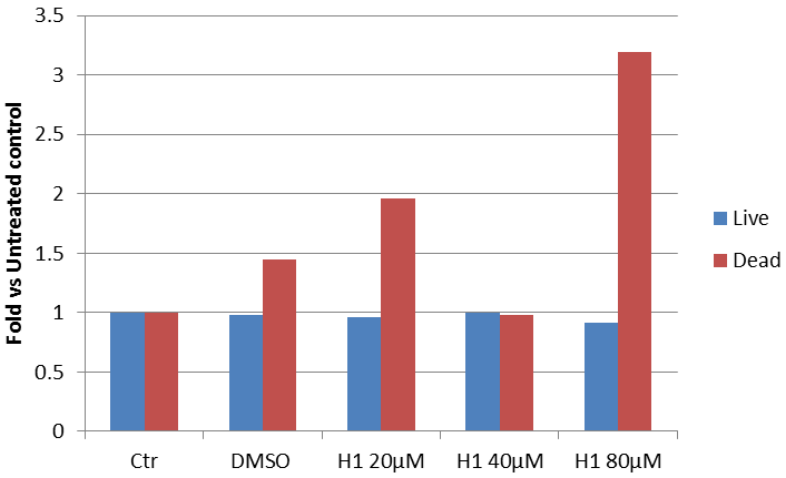


Figure 19. Cell viability assay evaluated by Trypan blue dye exclusion method of PC3 cell line exposed to different concentration of extract fraction.

In the final phase, Prof. Baker and his group will purify this extract though HPLC and the structure of this metabolite will be determined using NMR spectroscopy. After the final round of purification and structure elucidation the crude compound will be sent to us for final characterization. Unfortunately, due to lack of time the final phase of characterization will not be part of my thesis but I will be working on this in the final months of my Ph.D.

5. DISCUSSION

P.falciparum topoiosmerase 1B is a 104 KDa monomeric enzyme, composed of four structural domains each one with a particular function crucial for the protein activity, localization and interactions. Despite several attempts to express and purify the enzyme we were unable to produce an active *P.falciparum* protein. We decided to construct a chimeric enzyme containing different domains swapped into the human topoisomerase counterpart to understand their role in modulating the catalytic mechanism. In the present study, we have characterized the hTop1 (pf-N-terminal) chimeric enzyme constructed by swapping the N-terminal of *P.falciparum* Top1 into hTop1.

Introduction of pfTop1 N-terminal into hTop1 alters the enzymes function *in vitro* and *in vivo*. In detail, hTop1 (pf-N-term) displays relaxation activity slower than the wild type protein (Figure 11). Study of the cleavage kinetics with radio labelled oligonucleotide CL14 showed that the hTop1 (pf-N-term) has also a cleavage rate slower than hTop1. Indeed, the cleavage product in the case of the chimeric enzyme is almost absent till 15 minutes (Figure 12). However, cleavage kinetics with another radio labelled oligonucleotide namely CL14-U has shown that the hTop1 (pf-N-term) cleaves at the same rate as hTop1 and reach the same plateau value (Figure 13). Analysis of the religation rate indicates that the presence of the N-terminal domain of pfTop1 strongly reduces the catalytic activity (Figure 14).

In order to understand the phenotypes produced by the chimeric enzymes, a cell viability assay has been carried out using MBY3 yeast strain deficient for the DNA damage repair mechanism. The plot reported in figure 15 shows that the number of viable cells expressing hTop1 wild type decrease as a function of time indicating that an active hTop1 causes cell damage and consequently cell death. In case of the chimeric enzyme, we observed that there is a higher number of viable cells confirming that the chimeric protein is less active also in the cells and then producing a lower degree of DNA damage.

Several studies have indicated that the N-terminal 191-214 residues are required for DNA binding and enzyme processivity and play an important

role in controlling motion within the hinge region (Alsner et al., 1992; Redinbo et al., 2000). Indeed, the N-terminal 203-206 region and in particular Trp-205, has been shown to influence the control of DNA rotation, likely coordinating the function of the linker domain and/or nose cone helices (Chillemi et al., 2004). It is interesting to notice that tryptophan 205-209, following the sequence of the human enzyme, are also conserved in the pfTop1, however the lower dimension of inserted N-terminal domain of pfTop1 into hTop1 decreases the relaxation rate, the cleavage rate and almost abolishes the religation.

In conclusion, I demonstrated that the N-terminal of hTop1 has an important role in the DNA sequence recognition since the insertion of the N-terminal of pfTop1 into hTop1 decreases the cleavage rate and inhibits the religation reaction, highlighting the importance of the inter-domains communication in modulating the enzyme function. Further experiments are needed using different chimera and different DNA substrates to better understand the inter domains interplay. This study can open a new avenue for the investigation and correlation between the different domains within the protein and provide some insight for the development of species specific drugs.

The search for novel natural drugs having potent inhibitory effect against deadly diseases is always a greater demand from the scientific community. With this view in mind, I investigated the interaction of hTop1 with several metabolites derived from Antarctic invertebrates.

The geographical and evolutionary history of the Antarctica results in a unique isolated ecosystem rich in different kinds of fauna and organisms that, due to the extreme conditions, have developed specific defenses to survive, producing unique natural metabolites. Several studies have indicated that metabolites purified from Antarctic organisms have potent biological activities like antibacterial, antiviral, antiparasitic, antioxidant, antiproliferative and anti tumoral. Maschek et al. showed that palmadorin, from Antarctic nudibranch *Austrodoris kerguelenensis*, inhibits key enzymes involved in the JAK/STAT pathway in human erythroleukemia (Maschek et

al., in 2012). Recently, Baker and his group isolated and characterized two new tricyclic sesquiterpenoids shagenes from Antarctic organisms one of which is active against *Leishmania donovani*, the parasite causing visceral leishmaniasis, and does not display cytotoxicity against the mammalian host (von Salm et al., 2014).

In the present study, extracts derived from Antarctic organism which were sent to us by Prof. Baker from University of South Florida has been investigated as potential hTop1 inhibitors. In the first phase, a total of 750 extracts were tested against hTop1 and 94 extracts were identified to be responsible for inhibition. Further investigation revealed that there were two different kinds of inhibitors, reversible and irreversible inhibitors. Out of 94 extracts, 89 were found to be irreversible and 5 were responsible for reversible inhibition (table 4). We selected 1 out of the 5 reversible inhibitors for further characterization. This extract comes from the Antarctic sponge *Artimisina plumosa.* In the next phase, Prof. Baker and his group purified the extract through MPLC and sent us 18 fractions for further investigation (Figure 16). I identified 9 fractions out of 18 which were responsible for reversible inhibition. Time course experiment with the most concentrated fraction shows reversible inhibition of hTop1 at a concentration of 30µM (Figure 17). Inhibition effect of this extract fraction was also tested in PC3 prostate adenocarcinoma cell line in collaboration with Dr. Annalucia Serafino from National Research Council, Rome, Italy. The results show that the metabolite was able to affect cell viability at a concentration ranging from 80 µM to 200µM (Figure 18 and 19). Further experiments with crude extract are needed to confirm this result. Analysis of the partial purified extract resulted in the identification of the fraction responsible for inhibition of hTop1 and cancer cell proliferation.

In conclusion, I demonstrated that a natural metabolite derived from Antarctic invertebrate can be a potent and novel inhibitor of hTop1 and cancer cell proliferation. Further experiments are needed to characterize the metabolite and understand the complete mechanism of inhibition. Our study can be useful in the identification of new natural compound having therapeutic properties related to cancer therapy.

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7. CURRICULUM VITAE

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Conferences attended:

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International **EMBO** Conference on DNA Topoisomerase and DNA Topology from 17 – 21 September 2017 in Les Diablerets, Switzerland

Publications:

Zhenxing Wang Z, Ilda D'Annessa, Tesauro C, A Ottaviani, **Bini C Soren**, Jagadish Babu Dasari, Anil T, Paola Fiorani. The Human DNA Topoisomerase I Mutant Gly717Asp: high religation rate is not always associated with Camptothecin resistance. **(Manuscript Under review) - International Journal of Biological Macromolecules.**

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