DNA Topoisomerases of Leishmania: The Potential Targets for Anti-Leishmanial Therapy

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CHAPTER

DNA Topoisomerases of *Leishmania*: The Potential Targets for Anti-Leishmanial Therapy

Benu Brata Das, Agneyo Ganguly and Hemanta K. Majumder*

Summary

Protozoan parasites of the genus *Leishmania* cause severe diseases that threaten human beings, both for the high mortality rates involved and the economic loss resulting from morbidity, primarily in the tropical and subtropical areas. This ancient eukaryote shows variable genetic diversity in their life cycle, wherein DNA topoisomerases play a key role in cellular processes affecting the topology and organization of intracellular DNA. Kinetoplastid topoisomerases offer most attractive targets for their structural diversity from other eukaryotic counterpart and their indispensable function in cell biology. Therefore, understanding the biology of kinetoplastid topoisomerases and the components and steps involved in this intricate process provide opportunities for target based drug designing against protozoan parasitic diseases.

Introduction

Leishmaniasis is a disease complex caused by 17 different species of protozoan parasites belonging to the genus *Leishmania*. The parasites are transmitted between mammalian hosts by phlebotomine sandflies. There are an estimated 12 million humans infected, with an incidence of 0.5 million cases of the visceral form of the disease and 1.5 to 2.0 million cases of the cutaneous form of the disease. Leishmaniasis has a worldwide distribution with important foci of infection in Central and South America, Southern Europe, North and East Africa, the Middle East, and the Indian subcontinent. Currently the main foci of visceral leishmaniasis (VL) are in Sudan and India and those of cutaneous leishmaniasis (CL) are in Afghanistan, Syria, and Brazil. In addition to the two major clinical forms of the disease, VL and CL, there are other cutaneous manifestations, including mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (LR), and post-kala-azar dermal leishmaniasis (PKDL) that are often linked to host immune status. The number of cases of leishmaniasis is probably under estimated as leishmaniasis is a reportable disease in only 40 of the 88 countries where it is known to be present.[1] Although the global burden of leishmaniasis has remained stable for several years, the patterns of the disease change continiously. With increasing numbers of human immunodeficiency virus (HIV) coinfections, human migration, and resettlement, there is a possibility of resurgence of the disease.[1] Improved approaches to diagnosis, vaccine development, vector and reservoir control and new drugs for treatment are still required.

To make the situation even worse, some parasite strains have also developed resistance against the classical antimonial drugs, like sodium stibogluconate and megalumine antimonite. The second line of drugs, amphotericin B and pentamidines, although used clinically are very toxic.

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Therefore improved chemotherapy of leishmanial infection is still desirable and the need for new molecular targets on which to base the future treatment strategies is clearly justified. In search for such strategies DNA topoisomerases of *Leishmania* offer most attractive targets. The aim of this article is to provide an insight into the target based therapeutic approach against Leishmaniasis.

DNA Topoisomerases: The Wonder Enzyme

Topoisomerases are enzymes that use DNA strand scission, manipulation, and rejoining activities to directly modulate DNA topology. These actions provide a powerful means to effect changes in DNA supercoiling levels and allow some topoisomerases to both unknot and decatenate chromosomes. They are truly wonders, as in their presence, DNA strands can pass each other as if the physical boundaries between them have disappeared. They single handedly solve various topological problems for effective propagation of the genetic material. They are involved in replication, transcription, chromosomal condensation and segregation and many other vital cellular processes.[2] The immense interest in topoisomerase research in recent years derives not only from the recognition of their crucial role in managing DNA topology, but also from one major advance in the field. A wide variety of topoisomerase-targeted drugs have been identified, many of which generate cytotoxic lesions by trapping the enzymes in covalent complexes on the DNA. These topoisomerase poisons include both anti-microbials, anti-parasitic and anti-cancer chemotherapeutics, some of which are currently in widespread clinical use.

Classification of DNA Topoisomeases

Topoisomerases are divided into two classes, based primarily on their mode of cleaving DNA.[3] Type I DNA topoisomerases act by making a transient nick on a single strand of duplex DNA, passing another strand through the nick and changing the linking number by steps of one. Type II topoisomerases act by transiently nicking both strands of the DNA, passing another double stranded DNA segment through the gap and changing the linking number in steps of two with the help of ATP molecules.[3] A topoisomerase reaction has three general mechanistic steps i.e

i. Binding of an enzyme to the substrate DNA
ii. Cleavage by trans-esterification reaction accompanied by the formation of a transient phosphodiester bond between a tyrosine residue in the protein and one of the ends of the broken strand and subsequent strand passage through the break leading to change in the linking number
iii. Strand religation and release of the enzyme as the DNA is religated.

Under normal condition, the covalent enzyme DNA cleavable complexes are fleeting catalytic intermediates and are present in low steady state concentrations, which cells can tolerate. However, conditions that significantly decrease or increase the physiological concentrations of these breaks unleash a myriad of deleterious side effects, including mutations, insertions, deletions and chromosomal aberrations.[4] Thus all topoisomerases are fundamentally dualistic in nature, catalyzing essential cellular reactions and possessing an inherent dark side capable of inflicting great harm to the genome of an organism. For these reasons DNA topoisomerases have been recognized as potential chemotherapeutic targets for antitumours and antiparasitic agents.[5,6]

DNA topoisomerases can be classified into three evolutionary independent families: type IA, type IB and type II. The *Escherichia coli* topoisomerase I and topoisomerase III, *Saccharomyces cerevisiae* topoisomerase III and reverse gyrase belong to the type IA or type I-5' sub-family as the protein link is to a 5' phosphate in the DNA. The prototype of type IB or I-3' enzymes are found in all eukaryotes and also in vaccinia virus topoisomerase I where the protein is attached to a 3' phosphate.[3] Though essentially similar in their action, these enzymes have a broader specificity than that of *E. coli* enzyme. Despite the differences in the mechanism and specificity between the bacterial and eukaryotic enzymes, the yeast DNA topoisomerase I has

been shown to functionally complement a bacteria mutant in DNA topoisomerase I.[7] A certain degree of divergence also exists in the substrate preference, cofactor requirement and subunit composition of different topoisomerase families. Type IA topoisomerases are able to relax only negatively supercoiled DNA and require magnesium and single-stranded stretch of DNA for their function. Topoisomerases IB, however, are able to relax both positively and negatively supercoiled DNA with equal efficiency and do not require a single-stranded region of DNA or metal ions for function.[8]

The type II family includes *E.coli* DNA gyrase, *E.coli* topoisomerase IV (par E), all known eukaryotic type II topoisomerases and archaic topoisomerase VI. Type II enzymes are homo dimeric (eukaryotic topoisomerase II) or tetrameric (gyrase), cleaving both strands of a duplex that changes in linking number in steps of two. The current mechanistic model for topoisomerase II catalysed reactions involves the binding of two segments of DNA: a G (gate) segment, which is cleaved in both strands by the enzyme with the formation of an ester bond between active tyrosines and 5'-phosphates in the DNA and a T (transport) segment, which is captured by an ATP operated clamp that passes through the enzyme-stabilized break in the G segment.[9,10]

The discovery of several new DNA topoisomerases has brought a deeper understanding of their important roles in living cells. The biological functions of DNA topoisomerases are deeply rooted in the double helical structure of DNA and the selection of double stranded DNA as substrate has set the stage for their entrance.[11] Broad classifications of the different types of topoisomerases in different organisms are represented in Table 1.

Because DNA topoisomerases play key roles in cellular processes, affecting the topology and organization of intracellular DNA, it is important to define the physiological functions and understand the molecular basis of their action. Moreover, beyond their normal cellular activities, these enzymes are proven molecular targets for clinically useful anti-tumor[12-14] and anti-microbial drugs.[15-17] In this context work on topoisomerases from the parasites has been a growing focus of interest.

Toxic chemotherapy and increasing drug resistance of some parasite strains to classical drugs along with coinfection of *Leishmania* with HIV, have made them a severe threat to public health in developing countries. Development of vaccines is still under trial and improved therapy desirable.

Table 1. Classification of type I and type II DNA topoisomerases from different species

Subfamily	Representative Members
IA	Bacterial DNA topoisomerase I & II
	Yeast DNA topoisomerase III
	DNA topoisomerase IIIα and III
	Mammalian DNA topoisomerase IIIα and III
IB	Vaccinia and Pox virus monomeric topo I
	Kinetoplastida bi-subunit topoisomerase I
	Mammalian mitochondrial topoisomerase I
	Eukaryotic monomeric topoisomerase I
IIA	Bacterial gyrase, DNA topoisomerase IV
	Phage T4 DNA topoisomerase
	Yeast DNA topoisomerase II
	Drosophila DNA topoisomerase II
	Mammalian DNA topoisomerase IIα and IIβ
IIB	*Sulfolobus shibate* DNA topoisomerase VI
	(subunit A homologous to yeast SP011)

Topoisomerases of Kinetoplastid Parasites

Type I DNA Topoisomerase

Type I DNA topoisomerases were isolated from *L. donovani*,[18-19] *Trypanosoma cruzi*[20] and *Crithidia fasciculate*.[21] The purified active enzymes (65 - 79 kDa) were ATP- independent and found to be sensitive to topoisomerase I specific inhibitor, camptothecin.[21] Although immunolocalization studies for *C. fasciculata* topoisomerase I showed that it is situated in the nucleus rather than the kinetoplast,[21] it has been demonstrated in trypanosomes that camptothecin treatment induces kDNA minicircle cleavage.[22] This observation suggests a possible existence of topoisomerase I in the kinetoplast of trypanosomes.

The first DNA sequence of a topoisomerase I-like gene from the kinetoplastid, *L. donovani* was reported by Broccoli et al, 1999. The deduced amino acid sequence of this gene showed an extensive degree of homology with the central core DNA binding domain of other eukaryotic type IB topoisomerases, including several conserved motifs but having a variable C-terminus. The conserved active site motif SKXXY was absent in the deduced amino acids sequence. The over-expressed protein in *E. coli* failed to show any relaxation activity in vitro or complement a mutant deficient in topoisomerase I activity.[23]

Type IB enzymes are the sole targets for a class of anti-tumor agents, camptothecins.[24] Type IB activity has been purified from a number of kinetoplastids.[25] The difference in the sensitivity of kinetoplastid topoisomerase I for camptothecin,[26] prompted the search for topoisomerase I sequence from kinetoplastid parasites which uncovered the existence of unique topoisomerase I from these parasites.[27]

All eukaryotic type IB topoisomerases are monomeric and consist of four domains.[2] The unconserved amino terminal domain contains putative signals for nuclear localization of the enzyme and is highly sensitive to proteolysis and dispensable for in vitro activity.[28] The largest core domain is essential for enzyme activity and shows high phylogenic conservation, particularly in the amino acid residues interacting closely with DNA. The third domain is known as the linker, which is poorly conserved and is variable in length. Finally, the carboxy terminal domain is highly conserved and contains the SKINYL motif. Cleavage occurs by trans-esterification reaction involving nucleophilic attack by an active site tyrosine (Tyr 723 in human Topo I) on a DNA phosphodiester bond resulting in the formation of a covalent DNA 3' phosphotyrosyl linkage. In religation phase a similar trans-esterification reaction involves attack by the free DNA 5' hydroxyl that releases the enzyme from DNA.[29,30]

Starting from bacteria to human to viruses, topoisomerases I are encoded by a single gene that contains the highly conserved DNA-binding and catalytic domains on a single peptide. But in kinetoplastid parasites, topoisomerase I is encoded by two genes, which associate with each other to form a hetero-dimeric topoisomerase I enzyme within the parasite. Emergence of the bi-subunit topoisomerase I in the kinetoplastid family have brought a new twist in topoisomerase research related to evolution and functional conservation of type IB family. Genetic analyses identify a gene for a large subunit, namely LdTOPIL, on *L. donovani* chromosome 34, encoding for a 636-amino acid polypeptide with an estimated molecular mass of 73 kDa. This subunit is closely homologous to the core domain of human topoisomerase I. The gene for the small subunit LdTOP1S encoding a 262 amino acid polypeptide with a predicted molecular mass of 28kDa, in turn is found on the *L.donovani* chromosome 4. The small subunit contains the phylogenetically conserved "SKXXY" motif placed at the C-terminal domain of all type I DNA topoisomerases, which conserves a tyrosine residue playing role in DNA cleavage (Fig. 1A). LdTOPIL shows about 54% identity with core subdomain of human topoisomerase I but less than 22% identity with the linker and the C-terminal domain. On the other hand, LdTOPIS shows 43.5% sequence identity with the C-terminal domain of human topoisomerase I, including alignment of conserved sequences surrounding the catalytic tyrosine residue. LdTOPIL also deviates significantly from human topoisomerase I at loop regions bounded by LdTOPIL residues Pro62-His63, Asp114-His 118 and Pro 341-Asp 342

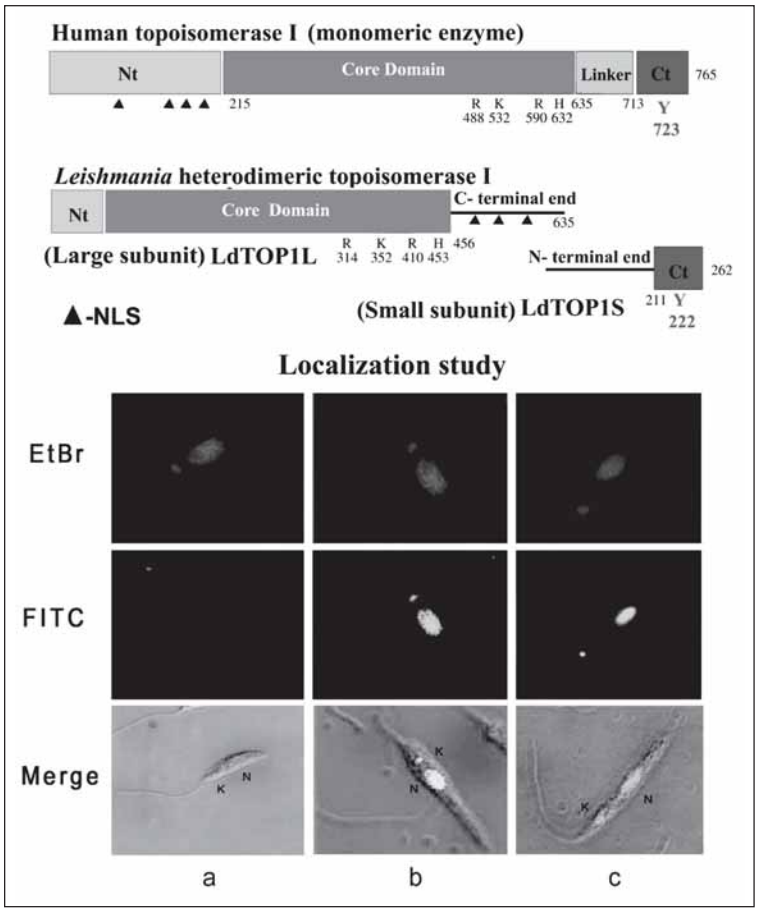

Figure 1. A) Schematic representation of the domain organization of monomeric human topoisomerase I and *Leishmania donovani* heterodimeric topoisomerase I. The domains are represented in different colored shades. Nt, N-terminal domain; Ct, C-terminal domain, NLS, Nuclear localization signal. Conserved residues are indicated in the figure. B) Immunocytochemical localisation with anti-LdTOP1L and LdTOP1S antiserum using fluorescent detection methods. Late log phase *L. donovani* promastigotes were fixed. No fluorescence was observed when preimmune serum was used as primary antibody and FITC-tagged secondary antibody (Panel a) as described by Das et al.[31] Panel b, same as Panel a, but probed with anti-LdTOP1L. Panel c, same as panel a, but probed with anti-LdTOP1S primary antibody. Parasite cells were also stained with ethidium bromide to locate the nucleus and kinetoplast and the area of the overlapping FITC and ethidium bromide (EtBr) stain are shown in merged pictures. Cells were viewed at an original magnification of 100 X under a Leica DM IRB inverted microscope. The nucleus (N) and kinetoplast (K) are indicated. Reproduced from reference 31.

which do not share the conserved sequences. Overall, this similarity indicates that the structure and catalytic machinery of the two enzymes are highly conserved, despite the fact that one is monomer and other is heterodimer (Fig. 1A).

Das et al,[31] described for the first time the in vitro reconstitution of the two recombinant proteins LdTOP1L and LdTOP1S corresponding to the large and small subunits. The proteins were purified from bacterial extract and the activity was measured by plasmid DNA

relaxation assay. LdTOP1L and LdTOP1S form a direct 1:1 heterodimer complex through protein-protein interaction. Under standard relaxation assay condition (50 mM KCl and 10 mM Mg^{2+}) reconstituted enzyme (LdTOP1LS) showed reduced processivity as well as 2 fold reduced affinity for DNA compared to eukaryotic monomeric rat liver topoisomerase I. Cleavage assay at various salt concentrations reveal that camptothecin (CPT) enhanced the formation of "cleavable complex" at low salt. Interaction between the two subunits leading to the formation of an active complex could be explored as an insight for development of new therapeutic agents with specific selectivity.

This observation leads to the concept that, non covalent interaction of both subunits is necessary for the activity. This was further evidenced from the charge difference of the two subunits. LdTOPIL has pI of 9.47 while that of LdTOP1S is 5.27. This charge difference clearly shows that these individual subunits are unstable until they interact with one another in the presence of salt. Recent findings by Das et al,[32] reveal that deletion of 99 amino acids from the N-terminus of LdTOP1L results in a protein which failed to interact with the smaller subunit. This could be attributed to the presence of many polar residues in this region. Polar interactions are common between the subunits of heterocomplex proteins. The overall charge difference for the large and small subunits in conjunction with the unusual salt sensitivity of the parasite protein suggests that ionic interactions are important for holding the subunits together.

Moreover it was established that silencing of one subunit in *T. bruci* causes the coordinate loss of both subunits of DNA topoisomerase I as well as results in a rapid reduction in the synthesis of both DNA and RNA of kinetoplastid parasites.[33]

Das et al,[34] also reveals that deletion of 39 aminoacids from the N-terminus of LdTOP1L results in a protein with decreased cleavage activity and sensitivity to CPT. These data argued in the favor of the interpretation that N-terminal amino acids of the large subunit regulates DNA dynamics during relaxation by controlling noncovalent DNA-binding or by coordinating DNA contacts by the other parts of the enzyme.

Davies and his co workers[34] have made a 2.27Å crystal structure of an active truncated *L. donovani* TOPIL/TOPIS heterodimer bound to nicked double stranded DNA in the presence of vanadate. The vanadate forms covalent linkages between the catalytic tyrosine residue of the small subunit and the nicked ends of the scissile DNA strand. This study reveals that arginine 410 residue of LdTOPIL (Arg 590 in human topoisomerase I) activates tyrosine 222 residue of LdTOPIS (Tyr 723 in human topoisomerase I) for attack on the scissile phosphate group, with water acting as a specific base. Moreover, it was also observed that Lys 352 of LdTOPIL (Lys 532 in human topoisomerase I) acts as the general acid in the cleavage reaction. Comparison of LdTOPILS to the structure of human topoisomerase I bound to DNA containing topotecan reveals that all of the amino acids that form the drug binding pocket are completely conserved between the two species (Fig. 1A).

Das et al, showed that LdTOPILS was localized in both nucleus and kinetoplast of *L. donovani* (Fig. 1B). The existence of multiple localization signals have been mapped in the larger subunits of *Trypanosoma* and *Leishmania* topoisomerase I[25,27] but no NLS has been found in smaller subunits of the enzyme. So it is likely that the subunits interact in the cytosol before nuclear and kinetoplast importation. But, whether the proteins perform separate functions in the cytoplasm is still unknown.

Type II DNA Topoisomerase

Topoisomerase II activities have been purified from various kinetoplastid parasites.[25] Topoisomerase II genes have also been cloned from *C. fasciculata, T. brucei, T. cruzi, L. donovani, L. infantum, L. chagasi,* and *Bodo saltans.*[25] The genes and proteins of the parasites were found to be smaller compared to higher eukaryotes. No gyrase like activity (capable of introducing supercoils into DNA) has been found and the enzymatic activities and the genes are more like other eukaryotic counterparts. The topoisomerase activity isolated from *C. fasciculata* was shown to be immunolocalized in kinetoplast[35] but the overexpressed proteins from *L. donovani* and *B. saltans* were found to be localized both in the nucleus and kinetoplast.[36,37] Although it can be

argued that the differences in cellular localization might be explained in terms of which epitopes were available for the recognition by different anti sera none the less the existence of another topoisomerase II sequence (hypothesized to be a mitochondrial topoisomerase II) cannot be dismissed. It is likely that replication of catenated kinetoplast DNA requires another topoisomerase activity.

Though all type II A topoisomerases are identical in one way that they change the linking number of DNA in an ATP-dependent manner, the eukaryotic type II enzymes are homodimers, while their bacterial counterparts like gyrase and topo IV are A_2B_2 tetramers; the B and A subunits being the N and C-terminal halves of their eukaryotic counterparts.[38] Certain phage type II topoisomerases are $A_2B_2C_2$ hexamers but they share the functional domains with other type II enzymes.[39,40] Interest in parasite type II topoisomerases and their genes and proteins gains impetus from the fact that they are the key enzymes involved in replication of the massive kinetoplast DNA network and RNAi of topoisomerase II leads to the progressive degradation of mitochondria in *Trypanosoma* . Kinetoplastid parasites diverged early in the eukaryotic evolution at the base of the evolutionary tree well before the emergence of kingdom metazoa. Inspite of having a similarity and identity of 31% and 23% with yeast topoisomerase II, LdTOP2 was found to complement a temperature sensitive mutant yeast strain.[41]

Just like other eukaryotic topoisomerase II, the parasite enzyme can also be divided into an N-terminal ATPase, a central DNA-binding and an unconserved C-terminal domain.[41-43] In spite of being unconserved, the nuclear localization signal and the dimerization domain of this homodimeric enzyme have been mapped in the C-terminus.[41] The C-terminus also contains a stretch of 60 amino acids not present in the human host. Therefore this region can be exploited to develop anti-leishmanial targets. The parasite enzyme has a greater affinity for DNA and was also stable at a very high salt concentration as compared to its human host.[42] These findings were quite consistent with the greater susceptibility of the parasite protein to the anti-topoisomerase II agents. This is because of the fact that an enzyme with more affinity towards DNA would perform more DNA cleavage and thus a greater chance of being trapped in that state by an anti-topoisomerase II drug.

The N-terminal 385 amino acids residues of LdTOP2 were found to possess the ATPase activity. Although the ATPase activity resides in the first 385 amino acid residues, only a larger protein was found to mimic the full-length enzyme kinetics in in vitro assay.[43] The study identifies specific amino acids like Asn65, Asn69, Asn96 and Asp130 of the parasite protein that are involved in the interaction with ATP and etoposide. In contrast, the ATPase domain of human topoisomerase IIα (1-453 amino acids) displays similar catalytic properties, in terms of ATP turnover, to that of the full-length enzyme, except for the fact that the smaller fragment (1-420 amino acids) fails to be hyperstimulated by DNA.[44] Most interestingly the ATPase activity of the N-terminal 385 amino acids of the parasite protein was also found to be inhibited by etoposide. Thus etoposide, in addition to being a poison for the parasite enzyme[42,43] is also a catalytic inhibitor of the enzyme.[43] The active site tyrosine implicated in DNA breakage and rejoining for *L. donovani* topoisomerase II has been mapped to be Tyr^{775}.[42] This tyrosine is the only residue in the parasite protein, which is involved in the trans-esterification reaction and is also homologous to the Tyr^{804} of human.[45] Surprisingly, the C-terminal truncation mutants of the parasite protein fail to be inhibited by etoposide[42] compared to the full length enzyme. Like the human enzyme, the core domain of LdTOP2 contains all the elements essential for sequence preference in protein-DNA interaction, but unlike the human enzyme, the C-terminus of the parasite protein plays an important role in the in vitro topoisomerase II cleavage reaction.

It was observed earlier that over expression of human N-terminal domain in yeast confers resistance to high concentrations of etoposide.[46] The observed phenotype was proposed to be due to the competition of the excess of the N-terminal domain with the full length enzyme for a limiting pool of inhibitor. So future challenge in the parasite topoisomerase II would be to develop drug resistant parasite strains and to see what causes this resistance and also to check what effect the individual domains of the enzyme have on the drug protein interaction in the context of the full-length enzyme.

Topoisomerases as Therapeutic Targets

Despite differences in catalytic mechanism and cellular functions, the critical feature of all topoisomerases is the DNA strand passage event. However, the ability to pass single or double-stranded segment of DNA freely through another comes with a heavy price; it requires enzymes that generate breaks in the genetic material. In an effort to maintain genomic integrity during this cleavage reaction, topoisomerases covalently attach to the newly generated DNA 3' (eukaryotic topoisomerase I) or 5' termini (all other topoisomerases via phosphotyrosyl bonds. Under normal circumstances, these covalent enzyme-DNA cleavage complexes are transient catalytic intermediates and are present in low concentrations and consequently, they are tolerated by the cell. However, conditions that significantly increase the physiological concentrations cause deleterious side effects, including mutations, insertions, deletions and chromosomal aberrations. Thus, all topoisomerases are fundamentally dualistic in nature. Although they catalyze essential reactions in the cell, they possess an inherent dark side capable of inflicting great harm to the genome of an organism.

Classification of Topoisomerase Inhibitors

Topoisomerase-targeting therapeutics currently in use act by trapping the covalent enzyme-DNA complexes of the first trans-esterification reaction. The known topoisomerase drugs can be divided into two classes, class I and class II.[5,47] The class I drugs have been referred to as 'topoisomerase poison' where as the class two drugs are referred to as 'topoisomerase inhibitors'. The class I drug act by stabilizing the covalent topoisomerase-DNA covalent complexes. These include bacterial gyrase inhibitors quinolones, eukaryotic topoisomerase I inhibitor camptothecin and topoisomerase II inhibitors amsacrine, doxorubicin, etoposide and teniposide. The class II drugs interfere with catalytic function of DNA topoisomerase without trapping the covalent complexes (Fig. 2). These classes of drugs include the coumermycin family of antibiotics that act on bacterial gyrases, the eukaryotic DNA topoisomerase II inhibitor suramin, fostriecin, merbarone and bis-dioxopiperizines. Several inhibitors of eukaryotic topoisomerase I have also been reported.

A major determinant of cytotoxicity for the class I drug is the conversion of a latent single or double stranded break in a drug-topoisomerase-DNA complex into an irreversible double stranded break. Replication is the key cellular process that drives this conversion in case of the topoisomerase I drug camptothecin. However, for class II topoisomerase II drugs, processes other than replication might also be involved. Cell killing by class II topoisomerase II drugs may involve cell cycle progression through mitosis. Traversing of eukaryotic cells through mitosis in the absence of functional DNA topoisomerase II can lead to aneuploidy and chromosomal breakage. For class I drug, cytotoxicity increases with increasing cellular level of target enzyme where as for class II drugs opposite is true. Thus increased levels of topoisomerases render cells hypersensitive to enzyme poisons but resistant to inhibitors. Conversely, decreased enzyme levels render cells resistant to poison but hypersensitive to inhibitors.

Topoisomerases as Targets for Anti Parasitic Agents

Sodium stibogluconate and ureastibamine, the two most potent and therapeutically used antileishmanial drugs have been reported by this laboratory to be specific inhibitors of *L. donovani* DNA topoisomerase I.[48] Pentavalent antimonials, also used as antileishmanial drugs, have been found to stabilize cleavable complex with an ED_{50} of 16.7 μg / ml and 209.5 μg / ml for wild type and resistant strains respectively.[49]

CPT, a plant alkaloid, an important class of antitumor agent[5] represents the best characterized topoisomerase IB inhibitor. It is reported to inhibit DNA topoisomerase I of *Leishmania* and *Trypanosoma*.[25] CPT is an uncompetitive inhibitor that directly traps the topoisomerase I-DNA covalent complex and slows the religation step of the nicking closing cycle.[2] CPT also hinders or blocks DNA rotation, which is evidenced by the crystal structure of the ternary complex between human topoisomerase I (topo 70) covalently linked to the DNA and the

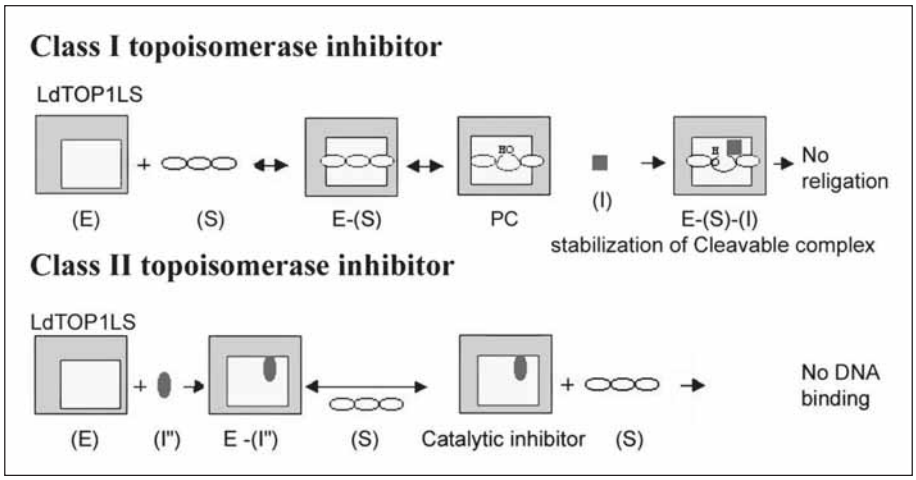

Figure 2. Schematic representation of mechanism of inhibition of bi-subunit topoisomerase I. Catalytic cycle of topoisomerase I is divided into DNA binding, cleavage and religation. A) CPT, a Class1 inhibitor, binds to the enzyme-DNA post-cleavage complex (PC), and subsequently inhibits religation step, and thus stabilizes the catalytic intermediate, (B) DHBA a pentacyclic triterpenoid, a Class II inhibitor, binds in the catalytic site of the enzyme and prevents binding of the enzyme with the DNA. E- is reconstituted bi-subunit *L. donovani* topoisomerase I (LdTOP1LS), where the green box represents the DNA binding large subunit (LdTOP1L) while the white small box is the catalytic subunit (LdTOP1S) harbouring SKXXY motif, S- substrate DNA, E-(S)- enzyme substrate complex, (I)- CPT, (I")-DHBA. A color version of this figure is available online at www.eurekah.com.

CPT derivative topotecan.[2] Recent finding reveals that a highly CPT resistant *L. donovani* strain (LdRCPT.160) developed by stepwise exposure to CPT induces point mutations (Gly 185 Arg and Asp325 Glu) in the large subunit (LdTOP1L) of the bi-subunit topoisomerase I. The mutant enzyme shows reduced activity as well as reduced sensitivity towards CPT.[50] The cytotoxicity of 9-substituted-10, 11-methylenedioxy analogs of camptothecin correlate well with cleavable complex formation in the nucleus and kinetoplast, and structural motifs have been identified that disproportionately increase toxicity to parasites, compared with mammalian cells. Sen et al,[51,52] has demonstrated that CPT induces programmed cell death (PCD) both in the amastigotes and promastigotes form of *L. donovani* parasite.

Structure-activity relationship studies with mitonafide have revealed that the compound inhibits both nuclear and mitochondrial topoisomerase of *Leishmania* with preferential targeting of the mitochondrial enzyme over the nuclear enzyme.[53] Anilinoacridines have recently been found to possess antiparasitic activity towards *Leishmania*, *Trypanosoma* and *Plasmodium* species. These compounds have been shown to induce protein associated DNA lesions in *L. chagasi* promastigotes. Linearization of kinteoplast DNA minicircles have also been reported in parasites treated with anilinoacridines at similar concentrations.[54] Members of the 9-anilinoacridine topoisomerase II inhibitors have also been shown to inhibit growth of *L. major* promastigotes and amastigotes.[55] 9-aminoacridines, that are reported topoisomerase II inhibitors and structurally related to the antileishmanial compound quinacrine and chlorpromazine, have shown anti leishmanial activity at concentrations in the range of 10-20 μM.[56]

For the last decade, our laboratory has been involved in the search of DNA topoisomerase targeted novel anti-leishmanial agents from various indigenous plants. Towards this goal we have isolated some compounds with profound antileishmanial effects. Amarogentin, isolated from *Swertia chirata*, was found to inhibit the catalytic activity of *L. donovani* DNA topoisomerase

I by preventing enzyme-DNA binary complex formation.[57] Administration of *L. donovani* infected golden hamsters with vesicular forms of amarogentin, liposome and niosomes, was found to be more effective than free amarogentin.[58] Indolyl quinoline, a biologically active synthetic compound, also acts as a dual inhibitor of *L. donovani* topoisomerase I and II.[59] Diospyrin, a bisnapthquinone isolated from *Diospyros montana,* have been reported to be a potent inhibitor of *Leishmania* topoisomerase I with no effect on topoisomerase II. Diospyrin requires a much higher concentration to inhibit calf thymus DNA topoisomerase I and has been reported to exhibit significant inhibitory effect on the growth of *L. donovani* promastigotes.[60] Roychoudhury et al, reported that dihydrobetulinic acid (DHBA), a derivative of betulinic acid that exhibits anti-HIV activity, is another excellent inhibitor of *Leishmania* DNA topoisomerase I and II[61] with the potential to become a lead therapeutic compound.[61] DHBA is a potent anti-leishmanial agent that induces apoptosis by primarily targeting parasitic topoisomerases. The structure of potential inhibitors of *Leishmania* topoisomerases are shown in Figure 3.

We have shown that the flavonoids quercetin and luteolin, isolated from *Vitex nigundo,* have potent antileishmanial effect. The flavonoids inhibited the growth of *L. donovani*

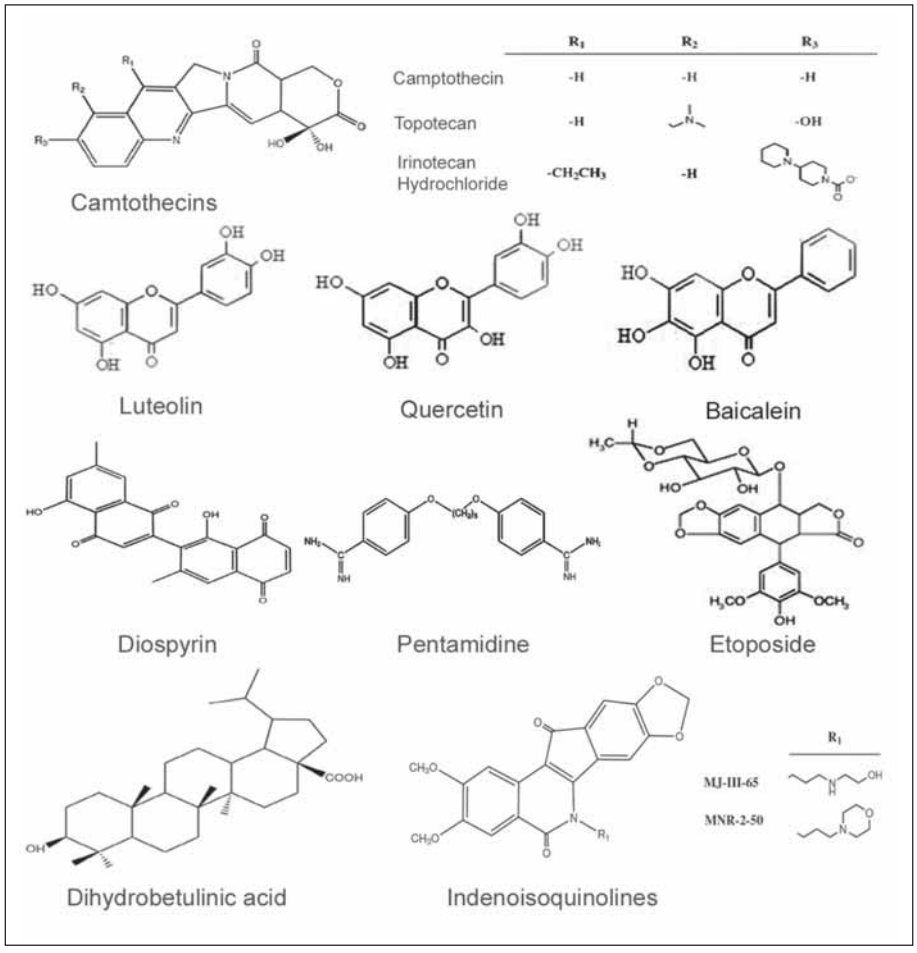

Figure 3. Structure of the potential *Leishmania* topoisomerases inhibitors.

promastigotes and amastigotes in vitro and also promoted topoisomerase II mediated linearization of kDNA minicircles. They arrested cell cycle progression in *L. donovani* promastigotes leading to apoptosis and reduced parasite burden in animal models.[62] Recently, Das et al,[63] described that naturally occurring flavones baicalein, luteolin and quercetin are potent inhibitors of the recombinant *Leishmania donovani* topoisomerase I. These compounds bind to the free enzyme and also intercalate into the DNA at a very high concentration (300 μM) without binding to the minor groove of DNA. The inhibition of topoisomerase I by these flavones is due to stabilization of topoisomerase I-DNA-cleavage complexes, which subsequently inhibit the religation step. Their ability to stabilize the covalent topoisomerase I-DNA complex in vitro and in living cells is similar to that of the known topoisomerase I inhibitor camptothecin (CPT). However, in contrast to CPT, baicalein and luteolin failed to inhibit the religation step when the drugs were added to preformed enzyme substrate binary complex. The most interesting part of the study reveals that baicalein and luteolin stabilize duplex oligonucleotide cleavage with CPT-resistant mutant enzyme LdTOP1Δ39LS lacking 1-39 amino acids of the large subunit.[32] This observation was further supported by the stabilization of in vivo cleavable complex by baicalein and luteolin with highly CPT-resistant *L. donovani* strain. Thus the interacting amino acid residues of *L. donovani* topoisomerase I may be partially overlapping or different for flavones and CPT.

Conclusion

Topoisomerase genes and proteins characterized from kinetoplastid parasite *Leishmania* appear to share many characteristics associated with their human homologues, but certain striking differences, including different enzyme activity requirements and different sensitivities to topoisomerase poisons provide insight for the development of topoisomerase-directed anti-parasitic therapeutics. It has been established by several studies that the inhibitors of topoisomerases convert these essential enzymes into intracellular proliferating cell toxins and thereby provide a good tool for preferentially killing of the highly replicative parasite cells within the host. The interaction of the enzyme with specific inhibitors and poisons screened from natural or synthetic sources will help in the quest to selectively target the topoisomerase-based replication apparatus as a means to therapeutically control the parasitic menace in the foreseeable future.

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