Review

DNA topoisomerases in apicomplexan parasites: promising targets for drug discovery

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The phylum Apicomplexa includes a large group of protozoan parasites responsible for a wide range of animal and human diseases. Destructive pathogens, such as \textit{Plasmodium falciparum} and \textit{Plasmodium vivax}, causative agents of human malaria, \textit{Cryptosporidium parvum}, responsible of childhood diarrhoea, and \textit{Toxoplasma gondii}, responsible for miscarriages and abortions in humans, are frequently associated with HIV immunosuppression in AIDS patients. The lack of effective vaccines, along with years of increasing pressure to eradicate outbreaks with the use of drugs, has favoured the formation of multidrug resistant strains in endemic areas. Almost all apicomplexan of medical interest contain two endosymbiotic organelles that contain their own mitochondrial and apicoplast DNA. Apicoplast is an attractive target for drug testing because in addition to harbouring singular metabolic pathways absent in the host, it also has its own transcription and translation machinery of bacterial origin. Accordingly, apicomplexan protozoa contain an interesting mixture of enzymes to unwind DNA from eukaryotic and prokaryotic origins. On the one hand, the main mechanism of DNA unwinding includes the scission of one—type I—or both DNA strands—type II eukaryotic topoisomerases, establishing transient covalent bonds with the scissile end. 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. The present review is an update on the new findings concerning topoisomerases in apicomplexan parasites and the role of these enzymes as targets for therapeutic agents.

**Keywords:** DNA topoisomerases; chemotherapy; apicomplexan protozoa; tropical diseases

1. INTRODUCTION

The phylum Apicomplexa includes parasites of major medical relevance such as \textit{Plasmodium} sp., \textit{Babesia} sp., \textit{Theileria} sp., \textit{Cryptosporidium} sp., \textit{Toxoplasma} sp. and others. All of them are unicellular organisms characterized by the lack of mobile structures and the presence of a representative organelle—the apicoplast, a relic chloroplast-like organelle of uncertain function that contains more than 500 functional predicted proteins and gives this phylum its name (Ralph et al. 2004; Morrison 2009). Species of genus \textit{Plasmodium}, \textit{Babesia} and \textit{Theileria} are zoonotic diseases transmitted by arthropod vectors. The \textit{Anopheles} mosquito is the vector of malaria, while \textit{Theileria} sp. and \textit{Babesia} sp. are currently transmitted by ticks of the \textit{Ixodidae} family. On the contrary, diseases caused by \textit{Toxoplasma} and \textit{Cryptosporidium} are not transmitted by arthropods. The contamination of foodstuffs, frequently raw or lightly cooked meat (containing \textit{Toxoplasma} bradyzoites) (Dorny et al. 2009) or water supplies with oocysts (resistant stages of \textit{Cryptosporidium}), is the major source of infective outbreaks (Fayer 2004). Once the micro-organisms enter their respective mammalian hosts, apicomplexan parasites undergo several development stages. Prior to erythrocyte invasion (exo-erythrocytic cycle), \textit{Plasmodium} and \textit{Theileria} reside within hepatocytes and lymphocytes, respectively, unlike \textit{Babesia} sporozoites, which directly infect host erythrocytes. By contrast, the life cycle of \textit{Cryptosporidium} occurs entirely in the host’s gastrointestinal tract, preferably in the brush border cells of the small intestine lining. This particular feature of \textit{Cryptosporidium} differs from the cyst-forming parasites like \textit{Toxoplasma}, whose sexual and asexual reproduction occur in different host species.

Malaria constitutes an important worldwide health issue given that more than 40 per cent of world population is affected by this disease. Malaria is endemic in over 100 countries, inhabited by 2400 million people. The World Health Organization estimated that there
were around 247 million cases of malaria in 2006, killing around one million people, many of them young children in Sub-Saharan Africa (http://www.who.int/mediacentre/factsheets/fs094/es/index.html). Plasmodium vivax or Plasmodium falciparum (the two most dangerous species of this genus) undergo asexual multiplication within erythrocytes, causing anaemia, fever, chills, nausea, a flu-like illness and, in severe cases, coma and death (Carter & Mendis 2002).

The clinical spectrum of babesiosis ranges from an asymptomatic infection to death (Vannier et al. 2008). As a consequence of parasite replication in the host, it often produces mild anaemia associated with few symptoms, but in more serious cases, it can progress to severe anaemia associated with numerous clinical manifestations, including high fever, chills, sweating, hypotension, pulmonary oedema, disseminated intravascular coagulation, haemoglobinuria and renal failure (Krause 2003).

Toxoplasma gondii is a highly prevalent parasite that infects one-third of the world’s population. Toxoplasma gondii is associated with congenital infections responsible for stillbirths and abortions. In addition, it causes neurological disorders (Costa da Silva & Longoni 2009), uveitis and systemic infections in immunocompromised patients (Tenter et al. 2000). In humans, T. gondii is acquired by consumption of lightly cooked meat, especially lamb and pork contaminated with bradyzoites. It can also be acquired by ingestion of oocysts containing sporozoites, which are the product of a sexual cycle in the intestine of the cat (Dorny et al. 2009).

Over the last 30 years, our concept of cryptosporidiosis has changed from that of a rare, largely asymptomatic disease, to an important cause of diarrhoea in humans and animals worldwide (Mosier & Oberst 2000; Chappell & Okhuyzen 2002). In humans, it accounts for up to 20 per cent of all the cases of childhood diarrhoea and is a potentially fatal complication in acquired immunodeficiency syndrome (AIDS) patients (Kosek et al. 2001). Cryptosporidium parvum oocysts are shed by infected animals or humans in the environment, and are responsible for water contamination and disease transmission.

Despite the fact that the role played by the host immune system in the resistance and healing of these diseases is well-established, no effective vaccine has so far been developed against any of the aforementioned protozoan infections. Therefore, prophylactic measures, hygienic conditions and easy access to effective medicines or combination therapies are the only means available to combat these devastating diseases. Chloroquine, mefloquine, quinine and primaquine remain the standard treatment for malaria in many countries, but resistant forms of the parasite are spread almost everywhere in the world (Winstanley & Ward 2006). With replacement of chloroquine, the number of combination sulfadoxine/pyrimethamine resistant parasites rose rapidly in most countries where it was introduced (Mita et al. 2009). New drugs such as the artemisinin-based combination treatments are clearly needed for first-line treatment of falciparum malaria in Africa and Asia (Eastman & Fidock 2009).

The combination of clindamycin and quinine is the current therapy of choice for babesiosis (Krause 2003). Treatment failures have been reported for this regimen, however, these have been mainly in patients infected with human immunodeficiency virus (HIV). Currently, drug therapy for ocular toxoplasmosis consists of a combination of antibiotics (Iaccheri et al. 2008; Commodoro et al. 2009). During the last decade, more than 100 different antibiotics and almost all known anti-protozoal agents have been tested against C. parvum with no or only limited success (Tzipori & Widmer 2008). The resistance of C. parvum to such a broad range of chemotherapeutic agents, some of which are effective against other apicomplexan parasites, might be related to the peculiar location of intracellular stages and to their almost complete sequestration from the host cytoplasm (Mead 2002). Paromomycin, a poorly absorbed aminoglycoside antibiotic, and, more recently, nitazoxanide were identified as promising agents for the treatment of cryptosporidium infections among AIDS patients (Tzipori et al. 1995). Both compounds showed efficacy in animal models, and paromomycin was found to be prophylactic in neonatal calves, lambs and goats (Theodos et al. 1998). More striking is the drastic reduction in the prevalence of opportunistic Cryptosporidium spp. infections associated with AIDS patients treated with highly active antiretroviral therapy (Zardi et al. 2005). Accordingly, there is an urgent need for the development of novel compounds to eradicate these infections with few or no side effects for the host.

2. DNA TOPOLOGY AND DNA TOPOISOMERASES

DNA supercoiling is important for DNA packaging within cells. Since DNA length can be thousands of times greater than that of a cell, its packaging within the nucleus is a difficult task. DNA supercoiling greatly reduces its volume and allows much more DNA to be packed in a very small volume. In prokaryotes, plec tonic-type DNA supercoiling prevails because DNA is organized into a relatively small, closed circular chromosome (Vologodskii & Cozzarelli 1994). However, in eukaryotes, DNA supercoiling occurs not only as a pletonic structure, but also as a solenoid—with the help of histones—which have been proved to be more effective for DNA compaction (Santisteban 1994). DNA supercoiling is also required for replication, transcription and genome recombination. Given that DNA must be unwound to allow the action of DNA/RNA polymerase complexes, the region placed ahead of those complexes must be positively unwound, whereas the lagging region behind the complexes must be rewound with negative supercoils. In addition, during replication, both freshly replicated DNA duplexes remain catenated and in order to ensure the integrity of the daughter DNA chains during cell division, they have to be completely separated (Peter et al. 1998). Topology provides mathematical tools for determining the state of DNA under different physical–chemical conditions. Supercoiling of a closed circular DNA molecule is produced by the torque generated by excess or deficit in the linking number (Lk) between the two DNA strands. Lk depends on two geometric properties, the twist (Tw) and writhe (Wr). Tw quantifies the number of times that a DNA strand is wound around the axis of the double helix, while Wr indicates the number of times that the trajectory of the axis of the double helix crosses itself when it has been projected.
onto a plane. Changes in Lk are always integers, unlike changes in Tw and Wr. Lk is always a constant provided by the sum of the changes in both parameters (Crick 1976).

It is necessary to break at least one of the DNA strands to introduce changes in Lk. This task falls to a group of enzymatic machines known as DNA topoisomerases (Top), a group of molecular machines discovered by James C. Wang in 1971 (Wang 1971). Top helps to solve topological problems of DNA by introducing single or double transient breaks and passing another duplex through the gate, thus resealing the passing molecule. The product of the activity of these enzymes is a more relaxed DNA, because its Lk is changed by one or more integers. Furthermore, some Top have additional activities, such as knotting/unknotting and catenation/decatenation (Wang 1996, 2002; Champoux 2001). A common feature of all Top is the presence of a tyrosyl residue in the active site of the enzyme. The tyrosyl residue is responsible for trans-esterification reactions with phosphate groups in the phosphodiester of the DNA backbone.

The coordinated three-step sequence performed by Top-manipulating DNA can be fuelled by the energy stored in the phosphodiester bond, which is conserved in the Top-DNA covalent intermediate, or by ATP hydrolysis. This feature, along with the number of DNA strands broken during the trans-esterification reaction, serves to classify Top into two different types (Corbett & Berger 2004). Type I Top (TopI) (EC 5.99.1.2) are monomeric (A) enzymes—with the notable exception of the heterodimeric (AB) enzymes described in Kinetoplastida—which introduce single transient breaks in DNA followed by passage and rejoining. TopI are ATP-independent enzymes that relax both positively or negatively supercoiled DNA. TopI are further classified into two families on the basis of the polarity of strand cleavage. TopIA family proteins introduce positive supercoils, decatenate single-stranded DNA and unwind supercoiled DNA through covalent bonding to the 5' end of the broken DNA strand, releasing a free 3'-OH strand. TopIB family proteins relax both positively and negatively supercoiled DNA, establishing temporary phosphotyrosine bonds with the 3' end of the broken DNA strand and releasing a free 5'-OH strand. The TopIA family includes bacterial TopI and TopII, archaeal TopI, bacterial and archaeal reverse gyrase and eukaryotic TopIII. The TopIB family includes eukaryotic TopI, TopV from hyperthermophilic Methanopyrus kandleri (Slesarev et al. 1993) and TopI from vaccinia poxvirus (Shuman 1998).

Unlike TopI, type II enzymes (TopII) (EC 5.99.1.3) are multimeric proteins that hydrolyse ATP to generate transient phosphotyrosine bonds at the 5' end of the two broken DNA strands, forcing the passage of another duplex through this break and rejoining (Bates & Maxwell 2007). TopII are not only able to relax positively supercoiled DNA (with the remarkable exception of prokaryotic DNA gyrases, which introduce negative supercoils into DNA), but also have extra knotting/unknotting and catenation/decatenation activities. Type II enzymes are organized into two families, TopIIA and TopIIB. The TopIIA family includes the homodimeric (A2) eukaryotic TopII, the bacterial heterotetrameric (A2B2) TopVI and DNA gyrase, and the heterohexameric (A2B2C2) Top IIA from T4 bacteriophages. These enzymes change the Lk by steps of ±2. On the other hand, the TopIIB family of proteins includes heterotetrameric (A2B2) enzymes that share most of the catalytic properties with bacterial type IIA and form double-stranded breaks with two base-pair overhangs at the cleavage site. TopIIB were discovered for the first time in extreme-thermophilic archaeabacteria Sulfolobus shibatai and they were called TopVI (Berger et al. 1994).

According to the annotations carried out in their corresponding genome projects, all apicomplexan parasites contain a typical eukaryotic TopIB as well as a putative TopIII-encoding gene but lack TopIA and TopV open-reading frames (ORF). On the other hand, multiple forms of TopIIA have been described in both the nucleus and the mitochondrion of eukaryotic organisms (no evidence of TopIIB ORFs have been annotated in the apicomplexan genome projects). Two forms of nuclear TopII—TopIIα and TopIIβ—participate in mitosis and/or meiosis of eukaryotic cells.

(a) Type I DNA topoisomerases

Because TopIA has no orthologues in eukaryotes, the current section is entirely dedicated to TopIB proteins. TopIB can relax both positive and negative DNA supercoils to achieve an entirely relaxed product. The ability to relax DNA supercoils allows this protein to play an essential role in transcription and DNA replication, since TopIB must relax the positive supercoils that accumulate ahead of the movement of polymerases, a task confined to TopII in bacteria. Relaxation occurs by a so-called ‘controlled rotation mechanism’ because the unbound 5'-OH is free to rotate before resealing. The catalytic cycle of TopIB consists of: (i) binding to DNA, (ii) a trans-esterification reaction between the hydroxyl group of Tyr residues placed at the active centre, which provides a transient covalent bond with the 3' end of the cleaved DNA strand, (iii) relaxation of DNA that is propelled by the superhelical tension, (iv) resealing of the phosphodiester bond using the energy stored in the DNA–TopIB complex, and (v) release of DNA restoring the integrity of the double helix (Stewart et al. 1998). Fragment complementation experiments (Stewart et al. 1997) and X-ray analysis of human TopIB (hTopIB) crystals have shown the existence of four structural and functional domains (Champoux 1998; Redinbo et al. 1998, 2000). The positively charged N-terminal domain is poorly conserved phylogenetically, but contains four putative nuclear localization signals (Mo et al. 2000). The core domain includes the DNA binding site and is essential for relaxation. It shows a high degree of phylogenetic conservation, including amino acid residues that are placed around the double helix. The core contains the conserved catalytic amino acids: Arg-488, Lys-532, Arg-590 and His-632 (catalytic tetrad) (Jensen & Svejstrup 1996; Stewart et al. 1996; Yang & Champoux 2001). The ability of TopIB to establish a temporal phosphodiester bond with DNA resides in a small reading frame (ORF). On the other hand, multiple forms of TopIIA have been described in both the nucleus and the mitochondrion of eukaryotic organisms (no evidence of TopIIB ORFs have been annotated in the apicomplexan genome projects). Two forms of nuclear TopII—TopIIα and TopIIβ—participate in mitosis and/or meiosis of eukaryotic cells.
(D’Arpa et al. 1988). This four-domain modular structure is fairly well conserved in Eukarya with the notable exception of Trypanosomatids (trypanosomes and leishmanias), where TopIB is a heterodimeric enzyme consisting of a large subunit closely homologous to the core domain of other TopIB, and a small subunit includes the C-terminal domain containing the tyrosil residue that plays a role in DNA cleavage (Bodley et al. 2003; Villa et al. 2003).

TopIB was first described from the murine parasite Plasmodium berghei by Riou and colleagues in 1986 (Riou et al. 1986), who purified and characterized the enzyme from infected erythrocytes. Later, the gene encoding the malarial (P. falciparum) enzyme (PfTopIB) was described: it consists of a monomeric protein of 104 kDa corresponding to a peptide of 839 amino acids encoded by a gene located on plasmoidal chromosome 5. This enzyme structurally resembles other topoisomerases, maintaining 42 per cent homology with the human enzyme (Tosh & Kilbey 1995). Three structural domains were described by the authors: (i) the N-terminus domain (134 amino acids), which is poorly conserved, (ii) a 500-residue long block of conserved amino acids with high level of homology to the core domain of the human protein, and (iii) the C-terminal domain, smaller than that of the hosts but with the conserved active tyrosine at position 798, which makes the enzyme fully active.

Sequence analysis showed two extensive tracts of additional amino acids within the core domain, one of 29–34 amino acids and another of 79 amino acids, whose function remains unknown (Cheesman 2000). Tosh and colleagues in 1999 showed that PfTopIB is developmentally regulated during the various stages of the Plasmodium life cycle. Northern analyses demonstrated that PfTopIB gene promoter is inactive in the ring forms, becoming active during the asexual intraerythrocytic cycle. High levels of PfTopIB mRNA are found during the trophozoite stage, but not in schizonts (Tosh et al. 1999).

Cryptosporidium parvum TopIB (CpTopIB) is codified by a single 1923 bp ORF (GenBankTM accession number XM-628497). The expressed polypeptide consists of 641 amino acids with a predicted molecular mass of 74.7 kDa and an estimated pl of 8.7, showing a 60 per cent sequence identity to hTopIB, 75 per cent identity to baker’s yeast (yTopIB) and 99.8 per cent identity to Cryptosporidium hominis (Ordoñez et al. 2009). The protein is much shorter than the human (765 amino acids) and baker’s yeast (769 amino acids) proteins, but contains all the recognizable domains of a canonical TopIB protein. CpTopIB contains a putative non-conserved N-terminal domain, a conserved core domain and a C-terminal domain, which shows a high level of homology with other eukaryotic C-terminal ends. This region includes a phylogenetically conserved ‘SKINY’ signature, in which Tyr-600 plays the role of a DNA-cleaving amino acid. The C-terminal domain is connected to the core by a poorly conserved putative coiled-coil linker.

Multiple alignment analyses with putative Apicomplexan TopIB proteins, as annotated in their corresponding genome project databases, show a great variability in length and predictable molecular weight. In line with this, the largest TopIB proteins are those from Theileria annulata, T. gondii and P. falciparum (953, 999 and 893 amino acids, respectively), whereas those from C. parvum and Babesia bovis (653 and 701 amino acids, respectively) are closer in size to the human enzyme (765 amino acids). Since the TopIB C-terminal end and the ‘linker’ domains (which maintain a uniform size across apicomplexan despite their differences in amino acid composition) are highly conserved in size in all species, the major alterations in size are owing to the ‘core’ and especially the N-terminal end domains. Sequence comparison shows a high degree of phylogenetic conservation in the three putative subdomains constituting the central core region, with inserts of variable size and unknown function in the Plasmodium recombinant protein (Tosh & Kilbey 1995) and also with the predictable translations of Th. annulata and T. gondii TopIB ORFs. However, greater variability is observed in the N-terminal-end region of these proteins in both size and amino acid sequences. The N-terminal regions of Th. annulata and T. gondii have a length of 308 and 338 amino acids, respectively, while B. bovis and P. falciparum have 116 and 156 amino acids, respectively, and C. parvum is the shortest, with only 57 amino acids.

Eukaryotic-type TopIB from parasitic protozoa are among the most evolutionarily remote DNA-cleaving proteins. The eukaryotic subfamily TopIB branched earlier than kinetoplastids from a common ancestor and includes most unicellular—Apicomplexan protozoa, yeasts and fungi—and pluricellular organisms with nuclear and mitochondrial operating enzymes. Within the Apicomplexa phylum, the Acanthoecidida class is composed of, among others, Haemosporidian (genus Plasmodium) and Piroplasmidia (genus Babesia and Theileria) protozoans. On the other hand, the class Coccidia contains the suborder Eimeriorina, which is represented by Cryptosporidium and Toxoplasma genus. The phylogenetic tree from figure 1a illustrates the good likeness between TopIB homology and phylogenetic categories. The position of kinetoplastids in this tree deserves special mention. Unlike the TopIB proteins described so far, which conserve the catalytic ‘tetrad’ in a single protein, trypanosomatid’s TopIB divide the active amino acid residues into two peptides that are post-translationally assembled. Previous phylogenetic analysis carried out with the gene encoding the large LdTopIB subunit and other eukaryotic TopIB sequences has shown that this branch occurred very early in evolution (Broccoli et al. 1999). This early divergence was caused by the absence of the C-terminal domain containing the tyrosine-cleaving residue, which was found later on in a separate chromosome of the leishmanial genome.

(b) Type II DNA topoisomerases

Owing to the lack of evidence of TopIIB-encoding genes in eukaryotic genomes, we will describe only the type IIA, which will be referred to as TopII hereafter. TopII are enzymes that need ATP energy for cutting both DNA strands and rescaling. Unlike TopI, this family of enzymes not only relaxes DNA, but also has additional catenation/decatenation and knotting/unknotting activities required for chromosomal segregation prior to anaphase, which is an essential prerequisite for normal
Figure 1. (a) Phylogenetic tree carried out using the amino acid sequences of type I DNA topoisomerases from apicomplexan parasites and other organisms annotated into the GenBank database. (b) Same as (a), but using the amino acid sequences of type II DNA topoisomerases. (c) Same as (a,b), but using the amino acid sequences of DNA gyrases: (i) gyrA, (ii) gyrB. The phylograms are displayed on TREEVIEW using the tree produced by CLUSTALW. The evolutionary scale bar is shown on the left; it indicates the relative distance on the tree in arbitrary units.

mitotic development. Phylogenetically, TopII monomers are homologous in structure, but their oligomeric status can vary between species. In contrast to the bacterial TopII, eukaryotic TopII are homodimers. Each subunit is formed by the fusion of domains corresponding to the GyrB/ParE (N-terminal end) and GyrA/ParC (C-terminal end) of bacterial DNA gyrase and TopIV subunits, respectively (Champoux 2001).

Eukaryotic TopII are composed of three structural domains (ATPase, B', A') that are connected by flexible hinge regions (Berget et al. 1996). These domains are present in a single polypeptide chain, unlike bacteria or bacteriophage T4, whose domains are split into different monomers. The ATPase domain is found at the N-terminal end of eukaryotic enzymes or the bacterial B subunit (GyrB or ParE for DNA gyrase and TopIV, respectively). The binding of ATP to the nucleotide-binding site causes the dimerization of the N terminal ATPase domains, whose opening and closing movements are regulated by the hydrolysis of ATP and the release of the product (Wei et al. 2005). The ATPase domain is followed by the core region, which consists of the B' domain and the N-terminal region of A' (Bjergbaek et al. 2000). The A' domain contains the active site tyrosyl residue required for catalysis and is called the 'breaking and resealing' domain. Eukaryotic TopII has a C-terminal hydrophilic domain that immediately follows the A' domain and whose sequence is similar in closely related species only. The C-terminal end is dispensable for biochemical activity, but it appears to have other intracellular functions, such as nuclear localization and interaction with other proteins (Reece & Maxwell 1991).

TopII are ATP-dependent enzymes that need ATP hydrolysis to perform their catalytic cycle and, therefore, they require Mg$^{2+}$ ions. Mg$^{2+}$ ions seem to promote the enzyme substrate interactions in at least two ways: (i) the direct reaction of enzyme-DNA cleavage and (ii) participation in the reaction and functions of ATPase providing Mg$^{2+}$ ATP substrate for enzymatic catalysis. Therefore, TopII (as well as TopIA) contains a conserved domain of around 100 residues, called Toprim, characterized by the presence of an invariant glutaminate residue and an aspartate-x-aspartate (DxD) motif (Aravind et al. 1998).

Despite TopII having been identified as a promising drug target against apicomplexan-borne diseases, molecular characterization of type II Top encoding genes and proteins has been poorly exploited. At the time of this paper being written, only the TopII genes from and proteins has been poorly exploited. At the time of molecular characterization of type II Top encoding genes and proteins has been poorly exploited. At the time of this paper being written, only the TopII genes from Plasmodium and Cryptosporidium spp., contain two gene encoding gyrase genes, which are translated in the cytoplasm into the corresponding proteins. The functional first Apicomplexan parasites, with the remarkable exception of Cryptosporidium sp., contain two gene encoding gyrase genes, which are translated in the cytoplasm into the corresponding proteins. The functional backbone of Apicomplexan DNA gyrase is a heterotetramer $A_B^2$ (Dar et al. 2007; Raghu Ram et al. 2007). Like the Escherichia coli DNA gyrase, the C-terminal end of the $P. falciparum$ GyrA (PfGyrA) subunit contains the DNA breakage and joining domain, as well as the site for DNA wrapping, whereas the N-terminal end of the GyrB (PfGyrB) subunit contains the ATPase domain associated with the Mg$^{2+}$-binding Toprim region and interacts with the GyrA subunit to build the active tetramer. The PfGyrB monomer contains a unique 45 amino acid insertion region within the Toprim domain that is essential for its in vitro and in vivo functions (Dar et al. 2007). Both subunits are larger than their bacterial counterparts, and the N-terminal domain contains the elements driving the enzyme to the apicoplast (Harb et al. 2004; Khor et al. 2005) (figure 1c). DNA gyrase is a type II topoisomerase responsible for catalysing ATP-dependent negative supercoiling of DNA in prokaryotes. Although DNA gyrase is commonly found in prokaryotes, some recent reports show the existence of bacterial-like DNA gyrase activity in plants and apicomplexan protozoa that may be important for DNA replication and transcription of endosymbiotic organelles. The apicomplexan apicoplast possesses a 35 kb circular genome likely to be of photosynthetic algal origin. This organelle became essential for cell survival in the later stages of its evolution owing to the presence of important pathways for the biosynthesis of some compounds, such as fatty acids of type II isopentenyl diphosphate and haem group intermediates (McPadden & Roos 1999). Most of the genes of the apicoplast genome encode some tRNAs and rRNAs, but the transcription and replication processes of the apicoplast genome are dependent on nuclear genes. The nuclear genome of apicomplexan protozoan contains homologues of gyrA and gyrB genes, which are translated in the cytoplasm into the corresponding proteins. The functional $P. falciparum$ DNA gyrase is a heterotetramer $A_B^2$ (Dar et al. 2007; Raghu Ram et al. 2007). Like the Escherichia coli DNA gyrase, the C-terminal end of the $P. falciparum$ GyrA (PfGyrA) subunit contains the DNA breakage and joining domain, as well as the site for DNA wrapping, whereas the N-terminal end of the GyrB (PfGyrB) subunit contains the ATPase domain associated with the Mg$^{2+}$-binding Toprim region and interacts with the GyrA subunit to build the active tetramer. The PfGyrB monomer contains a unique 45 amino acid insertion region within the Toprim domain that is essential for its in vitro and in vivo functions (Dar et al. 2007). Both subunits are larger than their bacterial counterparts, and the N-terminal domain contains the elements driving the enzyme to the apicoplast (Harb et al. 2004; Khor et al. 2005) (figure 1c). DNA gyrase is a type II topoisomerase as drug targets Structural differences between protozoan and host enzymes or unique enzymes with no orthology in the host and involved in singular metabolic pathways are ideal targets for a rational chemotherapy approach. In addition, key enzymes showing differential expression patterns are also suitable for drug targeting when structural differences do not enable these enzymes to be pharmacologically distinguishable from those of the
host. Both considerations make apicomplexan Top interesting potential targets for chemotherapy. On the one hand, most protozoan apicomplexan parasites contain a unique type of 27–35 kb apicoplast DNA, which requires bacterial-type DNA gyrase for replication, thus providing a unique drug target absent in the hosts (McFadden & Roos 1999; Dahl & Rosenthal 2008; Fleige & Soldati-Favre 2008). On the other hand, the higher expression rate of TopI and TopII in rapidly growing parasites is more likely to induce the formation of DNA–enzyme complexes, which are ultimately responsible for parasite cell death.

(i) Targeting TopI
Since its discovery in 1970s, TopIB has been identified as an attractive target for therapeutic intervention in proliferative processes because of its strategic position in replication, transcription, recombination and DNA repair (Pommier et al. 2003). Owing to this pivotal role in preserving the fidelity of genetic information, its structure has remained phylogenetically conserved—with the remarkable exception of kinetoplastids (Balana-Fouce et al. 2006; Reguera et al. 2006); therefore, drugs usually do not discriminate between the invading parasite enzymes and those from the host (Forterre et al. 2007). Despite structural resemblances to the host, apicomplexan TopIB exhibited different expression rates during its life cycle, validating its therapeutic interest. Drugs targeting TopIB are classified into two groups: (i) compounds that stabilize the enzyme/DNA cleavage complex; class I inhibitors or enzymatic poisons and (ii) compounds that interfere with the enzymatic catalysis or class II inhibitors. The most striking class I compounds are CPT and its structural derivatives (Pommier 2006). CPT is a natural pentacyclic alkaloid, first isolated from Camptotheca acuminata, a tree from the Nyssaceae family that originates from China and Tibet (Wall et al. 1966). At present, CPT represents an amazing tool for topoisomerase research, but owing to its toxicity it was ruled out early as a chemotherapy agent (Pizzolato & Saltz 2003). However, CPT has led an extended series of anti-tumour compounds, some of which have been integrated into cancer chemotherapy since the late 1990s (ten Bakkel Huinink et al. 1997; Cunningham et al. 1998). There are few studies focused on the effects of TopIB inhibitors in apicomplexan protozoa. Studies carried out with recombinant P. falciparum (Bodley et al. 1998) and C. parvum (Ordoñez et al. 2009) TopIB showed that CPT promotes the formation of cleavable complexes in vitro. In addition, blood-stage forms of P. falciparum are killed at micromolar concentrations of CPT (Tosh et al. 1999). However, further in vivo studies are needed to validate this enzyme as a true chemotherapy target in apicomplexans.

(ii) Targeting TopII
Like TopIB, eukaryotic TopII has been proposed as a putative target for anti-parasitic drugs since the 1990s (Burri et al. 1996; Cheesman 2000). The main objective of most TopII inhibitors consists in the irreversible stabilization of ternary cleavable complexes formed during topoisomerization. One of the first groups of compounds that showed therapeutic potential against Plasmodium was derived from the 9-anilinoacridine backbone, and had potential anti-tumour activity owing to TopII inhibition (Chavalitshewinkoon et al. 1993). Since then, hundreds of acridine compounds have been synthesized, led by amsacrine (m-AMSA), a DNA intercalating agent with remarkable anti-neoplastic activity against leukaemia and lymphoma cells (Louie & Issell 1985).

Many 9-anilinoacridine analogues have been assessed as potential anti-protozoal agents. Among them, pyronaridine showed high anti-plasmodial activity in vitro and was subjected to advanced clinical trials in Africa and Thailand during the 1970s (reviewed by Fu & Xiao 1991). Pyronaridine, a 9-anilino-aza-acridine, has schizontocidal activity at submicromolar concentrations and inhibits the decatenation activity of plasmoidal TopII (Auparakkitanon & Wilairat 2000).

Epipodophyllotoxins, another group of eukaryotic TopII poisons, have also been evaluated on protozoan parasites. These compounds are semisynthetic derivatives of the natural product podophyllotoxin, which is a non-alkaloid toxin derived from root extracts of genus Podophyllum plants. Representative epipodophyllotoxins, etoposide (VP16) and teniposide (VM26), are clinically active against Kaposi’s sarcoma, testicular cancer, acute lymphocytic leukaemia and small-cell lung cancer (Anonymous 2000). Their mechanism of action is related to the formation of cleavable complexes, but they do not intercalate into DNA. Etoposide treatment of P. falciparum blood-stage parasites promoted cleavage of both nuclear and apicoplast DNA (Weissig et al. 1997). In addition, treatment of asexual stages of P. falciparum parasites with etoposide resulted in chromosomal cleavage at a single locus (Kelly et al. 2006) as well as the induction of an apoptosis-like cell death process (Meslin et al. 2007).

(iii) Inhibition of DNA gyrase
Apicoplast DNA gyrase of apicomplexan parasites has no orthologous in mammals, thus providing an attractive target for drug development. Quinolone-based antibiotics—fluoroquinolones—are potent drugs that target bacterial DNA gyrase—mostly in Gram (−) and TopIV in Gram (+) micro-organisms. Fluoroquinolones bind to GyrA and ParC subunits of DNA gyrase and TopIV, respectively, establishing ternary covalent cleavage complexes with DNA, which actively kill pathogenic bacteria (Drlica 1999). Ciprofloxacin (derived from 7-chloroquinoline, a byproduct of chloroquine) was the first fluoroquinolone described to inhibit the growth of P. falciparum (Divo et al. 1988). Early studies showed that ciprofloxacin treatment induced cleavage of apicoplast DNA in P. falciparum, but it did not appear to target nuclear DNA, which suggested that a second uncharacterized type II enzyme might drive the replication of apicoplast DNA in the malaria parasite (Weissig et al. 1997). Ciprofloxacin had no beneficial effects against Babesia experimental infections (Weiss et al. 1993) and was only moderately active against C. parvum (Woods et al. 1996), in part owing to the absence of DNA gyrase. However, it was very active against Toxoplasma. The treatment of T. gondii with ciprofloxacin led to a decrease in the apicoplast genome copy number during replication. However, these parasites
were able to continue the replication cycle, although they were unable to invade new host cells undergoing a ‘delayed death’ (Fichera & Roos 1997). Nevertheless, controversial results have been reported concerning whether the induction of delayed death in malaria parasites is a prerequisite for the fluoroquinolone mode of action. On the one hand, supratherapeutic concentrations of ciprofloxacin can cause immediate death, but when clinically relevant concentrations were used, evidence was seen of the induction of the delayed death phenomenon (Dahl & Rosenthal 2007). These facts suggest that the induction of delayed death in Plasmodium (Goodman et al. 2007) that may be related to the expression rate of DNA gyrase at different stages of the parasite life cycle. Trovafloxacin was shown to successfully inhibit the replication of T. gondii in a murine model of acute toxoplasmosis (Khan et al. 1996). On the other hand, the GyrB subunit of bacterial DNA gyrase/TopII is targeted by coumermycin Al (Khor et al. 2005) and novobiocin (Raghu Ram et al. 2007), two aminocoumarin compounds structurally resembling fluoroquinolones. In vitro tests have shown that novobiocin was able to inhibit PfGyrB ATPase activity, delaying the trophozoite to schizont conversion at micromolar concentrations.

3. CONCLUSION

Apicomplexan protozoan contain an unusual combination of Top enzymes that makes them particularly attractive for drug development. The presence of a second endosymbiotic organelle, the apicoplast (which is of prokaryotic algal origin), implies the existence of an extranuclear DNA that is replicated and transcribed by enzymes similar to those found mostly in bacteria, but that are encoded by nuclear genes. Therefore, in addition to the eukaryotic TopIB and II, apicomplexan have a DNA gyrase similar to that found in E. coli, but operating specifically in the apicoplast. These enzymes are excellent targets for various anti-bacterial agents including quinolones and coumarins.

Interestingly, the blockade of the biological activities of apicoplast does not result in immediate death of the parasite. By contrast, the inhibition of apicoplast DNA gyrase with anti-bacterial quinolones leads to a specific phenotype called delayed death, observed in all apicomplexan except Cryptosporidium, which lacks apicoplasts. The parasiticidal effect of these drugs is effective only during certain stages of the life cycle of these protozoa.

On the other hand, apicomplexan eukaryotic Top IIs operating in the nucleus might be able to be targeted by anti-cancer drugs, like CPT derivatives or etoposide. Thanks to molecular and docking studies, novel structural insights have been gained into both apicomplexan enzymes. These differences could be useful in the near future as a good source for the development of new leading compounds, reinforcing the hypothesis of their suitability as promising targets for anti-parasitic drug development. However, owing to the scant information about the effect of these compounds on experimental infections, there is a pressing need to perform systematic in vivo trials to evaluate the actual efficiency of drug treatments targeting TopIB, TopII and DNA gyrases. In conclusion, a major effort is needed to validate these enzymes as parasiticidal drug targets—but it is already clear that this field has great potential for rational drug design.

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