DNA Topoisomerases as Targets for Antiprotozoal Therapy

R.P. Bakshi and T.A. Shapiro*

Division of Clinical Pharmacology, Department of Medicine and Department of Pharmacology and Molecular Sciences; and Malaria Research Institute; The Johns Hopkins University, Baltimore, MD 21205, USA

Abstract: Diseases caused by parasitic protozoa present a health problem of immense magnitude, and there is an urgent need for safe and effective new therapies. DNA topoisomerases are clinically relevant targets for anti-cancer and anti-bacterial agents. Inhibitor studies on parasite topoisomerases have revealed that these enzymes have great promise as molecular targets for anti-parasitics, and have helped to dissect the basic biology of DNA topoisomerases in these organisms. This review provides a brief introduction to DNA topoisomerases and anti-topoisomerase drugs, and an overview of studies on protozoal DNA topoisomerases and their inhibitors.

Key Words: DNA topoisomerase; antiparasitic; poison; inhibitor; *Trypanosoma*; *Plasmodium*; *Leishmania*; protozoa.

INTRODUCTION

Infectious diseases caused by blood-borne parasitic protozoa are widespread and affect a significant proportion the world's population. African trypanosomiasis and Chagas disease, caused by *Trypanosoma* species, together account for approximately 20 million infections [1, 2], and some twelve million people suffer from leishmaniasis which is caused by *Leishmania* species that are closely related to the trypanosomes [3]. Outstripping all other protozoal diseases in its medical importance is malaria (caused by *Plasmodium* species). There are 300 million cases of malaria reported annually, and the World Health Organization estimates that 2.4 billion people- 40% of the world's population- are actually infected [4]. In Europe and the United States, where these parasites are much less commonly transmitted, closely related pathogens typically cause disease (*e.g.*, toxoplasmosis, cryptosporidiosis) in immunosuppressed individuals and are of considerable importance in veterinary medicine. Each of these diseases leads to substantial morbidity and mortality in infected populations. The antiquity of the drugs available (agents of choice include trivalent arsenicals and pentavalent antimonials), coupled with the well-documented threat of resistant parasites, has led to a growing realization of the need to identify novel molecular targets and therapies for parasitic infections.

The rational development of successful new anti-parasitic drugs must take certain factors into account. Most important is the choice of an appropriate target: the agent should interfere with a system whose proper function is essential for survival of the parasite. Secondly, there should be distinct and exploitable differences between the target and its counterpart (if any) in the host organism. These two factors determine the efficacy and specificity, and ultimately the success, of the drug. Furthermore, because parasitic diseases afflict such large numbers of people in areas that typically are economically underdeveloped, safety and low cost are additional critical factors.

*Address correspondence to this author at the Division of Clinical Pharmacology, Department of Medicine and Department of Pharmacology and Molecular Sciences; and Malaria Research Institute; The Johns Hopkins University, Baltimore, MD 21205. USA; Tel.: 410-955-1888; Fax: 410-955-2634; E-mail: tshapiro@jhmi.edu

Fortunately, in addition to their tremendous medical importance, the pathogenic protozoa are fascinating experimental subjects. Many important discoveries, including antigenic variation, sequence-directed bent DNA, and glycosylphosphatidylinositol (GPI) protein anchors, were made first in parasites. Indeed, it was Paul Ehrlich's work with trypanosomes and malaria parasites that led to the founding principles of modern chemotherapy: the molecular basis for drug action; rational drug design; and selective toxicity; that were recognized by the 1908 Nobel prize. The kinetoplastids (trypanosomes and leishmania) and malaria parasites are ancient eukaryotes. Their metabolic pathways may be rudimentary and inefficient, and they have structural features not seen in higher eukaryotes. Among the most distinctive of these features is their organellar (non-nuclear) DNA. As described below, the mitochondrial DNA in kinetoplastids and the apicoplast DNA in *Plasmodium* are unusual in their structure and sequence, and they have no counterpart in mammalian cells. The enzymatic machinery responsible for the synthesis and maintenance of these unique DNAs is therefore an attractive candidate for therapeutic intervention. Of these enzymes, the DNA topoisomerases are obvious targets. They are ubiquitous, essential for cell survival, and are the basis for the molecular mechanism of action of clinically useful antibacterial and antitumor drugs that are prescribed in multibillion-dollar quantities every year.

In this review we provide a brief introduction to the DNA topoisomerases and anti-topoisomerase drugs, and then examine the existing information on protozoal DNA topoisomerases and their inhibitors. For the latter, the discussion is confined almost entirely to the kinetoplastids and malaria parasites, where most work has been done.

DNA TOPOLOGY AND THE TOPOISOMERASES

In cells, the helical form of DNA poses structural and metabolic challenges that are topological in nature (DNA topology reviewed in refs. 5, 6). Two strands of DNA are twisted around each other to form a right-handed helix that contains about 10 base pairs per turn. Conventionally, the sense of this twist is considered positive. Supercoiling is the phenomenon of the helix axis coiling about itself. The sense

1389-5575/03 $41.00+.00

of supercoiling can be opposite to (negative), or the same as (positive), the twist of the duplex. A DNA double-helix whose ends are unable to freely rotate, such as covalently closed circular DNA, is subject to a fundamental topological constraint: the algebraic sum of the number of double helical turns (twist) and the number of supercoils is a constant, known as the linking number. The linking number of a DNA molecule cannot be altered without breaking one or both strands of the helix (much as a coiled telephone cord cannot be straightened without lifting and manipulating the handset). DNA isolated from natural sources is supercoiled negatively in mesophiles and positively in thermophiles.

Supercoiling offers many advantages: it reduces the effective volume of DNA and enables compaction of the genome, and it promotes the binding of proteins that locally unwind DNA and mediate various DNA metabolic processes. However, supercoiling may cause severe topological problems when the DNA needs to be accessed for various metabolic processes. Processes requiring tracking along the DNA molecule, such as transcription and replication, themselves may lead to progressive topological conditions that need to be resolved. Resolving these constraints invariably requires transient breaking of the double helix. Biological systems have overcome this problem through the use of activities known as DNA topoisomerases. These enzymes can modulate the topological state of DNA without altering sequence information therein. They catalyze a variety of reactions including DNA relaxation, knotting/unknotting and catenation/decatenation Fig. (**1**) and are involved in virtually every DNA transaction occurring in the cell (see refs. 7, 8 for review of DNA topoisomerases).

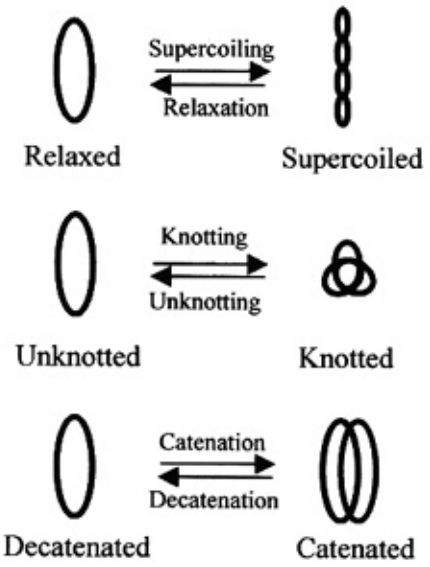


Fig. (1). Reactions catalyzed by DNA topoisomerases. Three topological interconversions of duplex DNA circles facilitated by DNA topoisomerases are shown. Type I and II topoisomerases can mediate all reactions; however type I enzymes require a nicked substrate for knotting/unknotting or catenating/decatenating activities. DNA gyrase is the only known topoisomerase that can introduce negative supercoils into DNA.

CLASSIFICATION OF THE TOPOISOMERASES

In essence, the DNA topoisomerases catalyze a sequential series of nicking, strand passing, and religating steps Fig. (**2A**). Based on catalytic mechanism, they are divided into two primary classes (Table **1**). Type I DNA topoisomerases create a single-strand nick in DNA, pass another DNA segment through this nick, and reseal it. The reaction is ATP-independent and changes substrate linking number in steps of one. Type II enzymes catalyze a double-strand break in DNA, through which another strand is passed. Topoisomerase II-mediated catalysis is ATP-dependent and leads to changes in substrate linking number in steps of two. For the DNA breakage-resealing processes, both types of enzyme rely on a transesterification reaction that entails the transient formation of a covalent phosphotyrosine bond between the enzyme and one cleaved end of its DNA substrate. This transient intermediate is not only obligatory in the chemical attack on the phosphodiester backbone, but it also serves to tether one of the free ends of the polymer and thus to prevent the uncontrolled unraveling of supercoils. The site of phosphotyrosine bond formation, at either the 3'- or the 5'-end of the nick in DNA, is a characteristic feature within various subclasses of the topoisomerases (Table **1**).

Type I DNA topoisomerases have been isolated and characterized from prokaryotes, eukaryotes and archaea, and are divided into Type IA and Type IB (Table **1**). These differ markedly in their primary sequence, catalytic requirements and mechanisms, and inhibitor susceptibilities. Type IA enzymes show sequence homology to *E. coli* topoisomerase I while *S. cerevisiae* topoisomerase I is the prototype of the IB class. Some eubacterial mesophiles and eukaryotes contain two type I activities: topoisomerase I and topoisomerase III, or topoisomerase IA and IB [9]. Extremophiles in both prokaryotic and archaeal kingdoms contain a distinct type I activity known as reverse gyrase. This enzyme possesses the ability to introduce positive supercoils into DNA in an ATP-dependent manner. *M. kandleri* has a unique type IB enzyme designated as topoisomerase V. This enzyme demonstrates homology to the integrase family of tyrosine recombinases and biochemical evidence suggests it may play a role in excision repair [10].

Type II enzymes have been extensively characterized from a variety of systems with *E. coli* DNA gyrase serving as the prototype. *E. coli* contains a second type II enzyme, termed topoisomerase IV. Although yeast and *Drosophila* possess just one type II enzyme, vertebrates are endowed with two isoforms of topoisomerase II, designated as topoisomerases IIα and IIβ. Recently, a topoisomerase VI has been described; this novel enzyme has homology with *S.cerevisiae spo11*, which is involved in meiotic recombination (Table **1**).

CELLULAR ROLES OF DNA TOPOISOMERASES

Topoisomerase biology has been the subject of several excellent reviews; the reader is referred to these for a more detailed examination of the topics discussed below [5, 8, 11, 12]. DNA topology influences various key processes

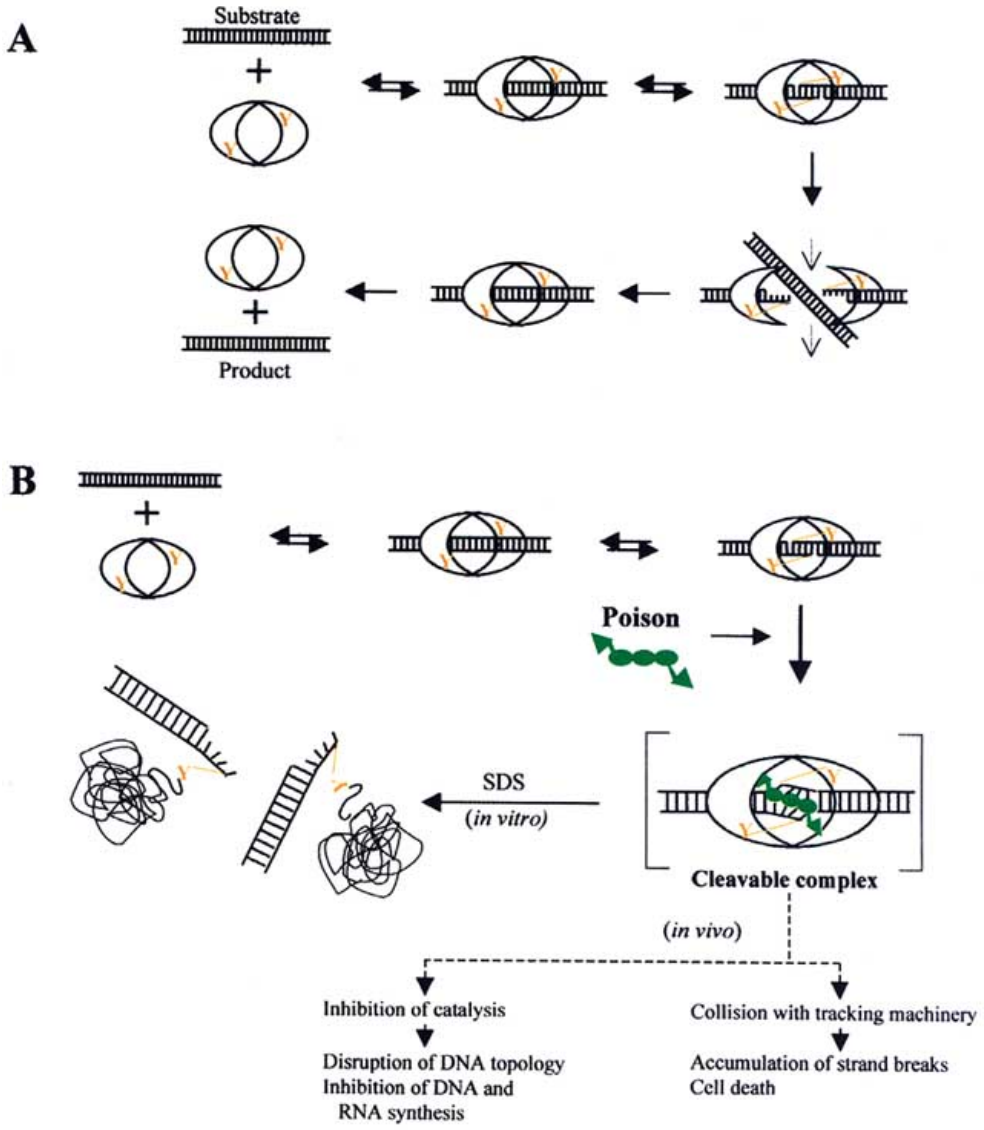


Fig. (2). **Mechanism of topoisomerase-mediated catalysis and inhibition. A.** Catalytic cycle of a topoisomerase is illustrated by a type II homodimer. The enzyme first binds reversibly to its DNA substrate to form a Michaelis complex. Transient breaks are then made in the DNA substrate, in which the 5'-ends of the DNA are tethered to the enzyme by a covalent phosphotyrosine linkage. Another DNA strand is passed through the double-stranded break, the break is resealed, and the enzyme dissociates from its product. For type II enzymes, ATP hydrolysis accelerates strand passage and is required for enzyme turnover. **B.** Mechanism of topoisomerase poisoning. Certain agents interact with the preformed enzyme-substrate complex and freeze it as ternary structure comprised of topoisomerase, DNA, and inhibitor. Disruption of this ternary complex (by alkali or denaturant *in vitro*, or by encounter with DNA processing machinery *in vivo*) leads to breakage of the DNA substrate with covalent attachment of a topoisomerase protein. *In vivo*, such DNA damage triggers autolysis.

including gene regulation, nucleic acid replication, chromosome condensation/decondensation, maintenance of genomic stability and chromosome segregation. Data from mutant or transgenic organisms, and from cells treated with topoisomerase inhibitors afford evidence that topoisomerases are essential for all these processes. Interestingly, in addition to reaction catalysis, the topoisomerases also contribute to the structural framework essential for the compaction of DNA polymers within the cell. While the multiplicity of type I and type II activities results in a certain amount of functional redundancy, each enzyme appears to have specific roles within the cell; to some extent these are best understood in prokaryotes.

Table 1. Classification of DNA Topoisomerases

Class	Features	Examples
Type I	Create single strand break in the DNA substrate linking number changes in steps of one ATP-independent	
Type IA	Tyrosine linked to 5' phosphate Require Mg^{+2} Utilize negatively but not positively supercoiled substrates	*E. coli* topoisomerase I and III *S. cerevisiae* toposiomerase III Human topoisomerase IIIα and IIIβ *S. acidocaldarius* reverse gyrase *M. kandleri* reverse gyrase[1]
Type IB	Tyrosine linked to 3' phosphate Do not require Mg^{+2} Can utilize both positively and negatively supercoiled substrates	*S. cerevisiae* topoisomerase I Human topoisomerase I Vaccinia virus topoisomerase I *M. kandleri* topisomerase V *P. aeruginosa* topoisomerase I
Type II	Create a double strand break in the DNA substrate Tyrosine linked to 5' phosphate Linking number changes in steps of two ATP-dependent	
Type IIA	Subfamily based on homology to *E. coli* DNA gyrase	*E. coli* DNA gyrase and topoisomerase IV S. *cerevisiae* topoisomerase II Human topoisomerase IIα and IIβ
Type IIB	Single member class Homologous to *spo11*	*S. shibatae* topoisomerase IV

[1]Only reported type I enzyme that is multimeric

In *E.coli*, topoisomerase I and DNA gyrase act in tandem to regulate supercoiling levels globally and during RNA synthesis in particular. Topoisomerase III in conjunction with topoisomerase IV plays a similar role during DNA replication, and topoisomerase IV is the primary mediator of the segregation of linked daughter chromosomes at the termination of replication. Mammalian cells may possess at least six distinct topoisomerases: topoisomerases I, IIIα, IIIβ, IIα and IIβ in the nucleus, as well as a newly described type I in the mitochondrion [13]. Insight into the function of these component enzymes has derived from a variety of experimental approaches, including inhibition, immunolocalization, and studies of cell-cycle dependence. Not surprisingly, the available evidence suggests that expression of these enzymes is carefully regulated at virtually every step of their production.

TOPOISOMERASES AS MOLECULAR TARGETS

Interest in DNA topoisomerases has been greatly spurred by the fact that numerous antibacterial and antitumor drugs target these enzymes. Compounds that inhibit topoisomerase activity may be usefully divided into the topoisomerase poisons and the topoisomerase inhibitors [14, 15].

Topoisomerase Poisons

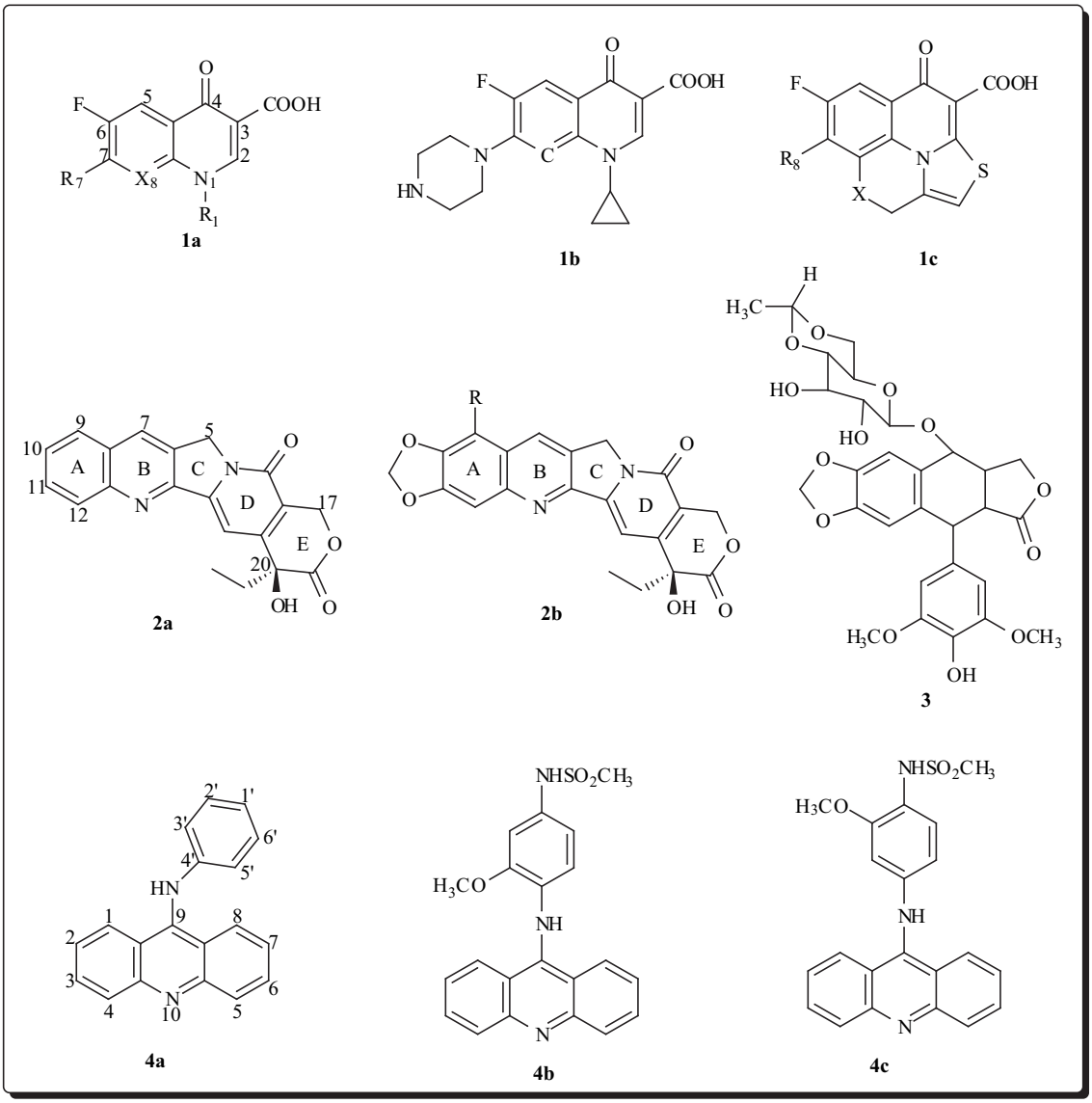
The poisons are (as the term suggests) a unique and exceedingly important subset of topoisomerase inhibitors that are available for both type I and II enzymes [14]. In general, they are highly selective against topoisomerases (and not other cellular targets), they are more potent than non-poison inhibitors at killing tumor or bacterial cells, and they comprise the clinically useful agents. In striking contrast to most inhibitors, which bind directly to their target enzyme, topoisomerase poisons bind to a preformed DNA-topoisomerase catalytic intermediate, to form a stable complex of enzyme, DNA substrate, and inhibitor Fig. (**2B**). This ternary structure has been termed a "cleavable complex" [16] because when rapidly disrupted (by ionic detergents or chaotropic agents) it yields a cleaved DNA molecule which is covalently linked via phosphotyrosine to one or two denatured topoisomerase proteins (for type I or II enzyme, respectively).

The ability to promote formation of cleavable complexes is the distinguishing characteristic of topoisomerase poisons. In the laboratory, this unusual phenomenon has proven extraordinarily useful for providing direct evidence of topoisomerase inhibition within the living cell (data not easily obtained with conventional inhibitors), as well as for structure-activity studies in drug development programs. Therapeutically, the formation of cleavable complexes contributes substantially to drug potency. Like conventional inhibitors, topoisomerase poisons block catalytic activity, which in turn disrupts the state of DNA supercoiling in the cell Fig. (**2B**). In addition to this, however, the mere presence of cleavable complexes is cytotoxic. When DNA tracking machinery collides with these immobile structures (*e.g.*, during DNA or RNA synthesis) there is an accumulation of DNA strand breaks in the cell, which triggers a series of events that lead to cell death. The interesting molecular details of this killing mechanism are the subject of active study in a number of laboratories [17]. Because the enzyme is a required component of the ternary cleavable complex, increased intracellular levels of topoisomerase increase cytotoxicity of the poisons. This is contrary to what is seen with conventional inhibitors, whose effect on overall catalytic capacity can be ameliorated by increased intracellular levels of the target enzyme.

Recognized poisons include the clinically useful antibacterial quinolones and fluoroquinolones (**1**; *e.g.*, ciprofloxacin **1b**); and the antitumor camptothecins (**2**),

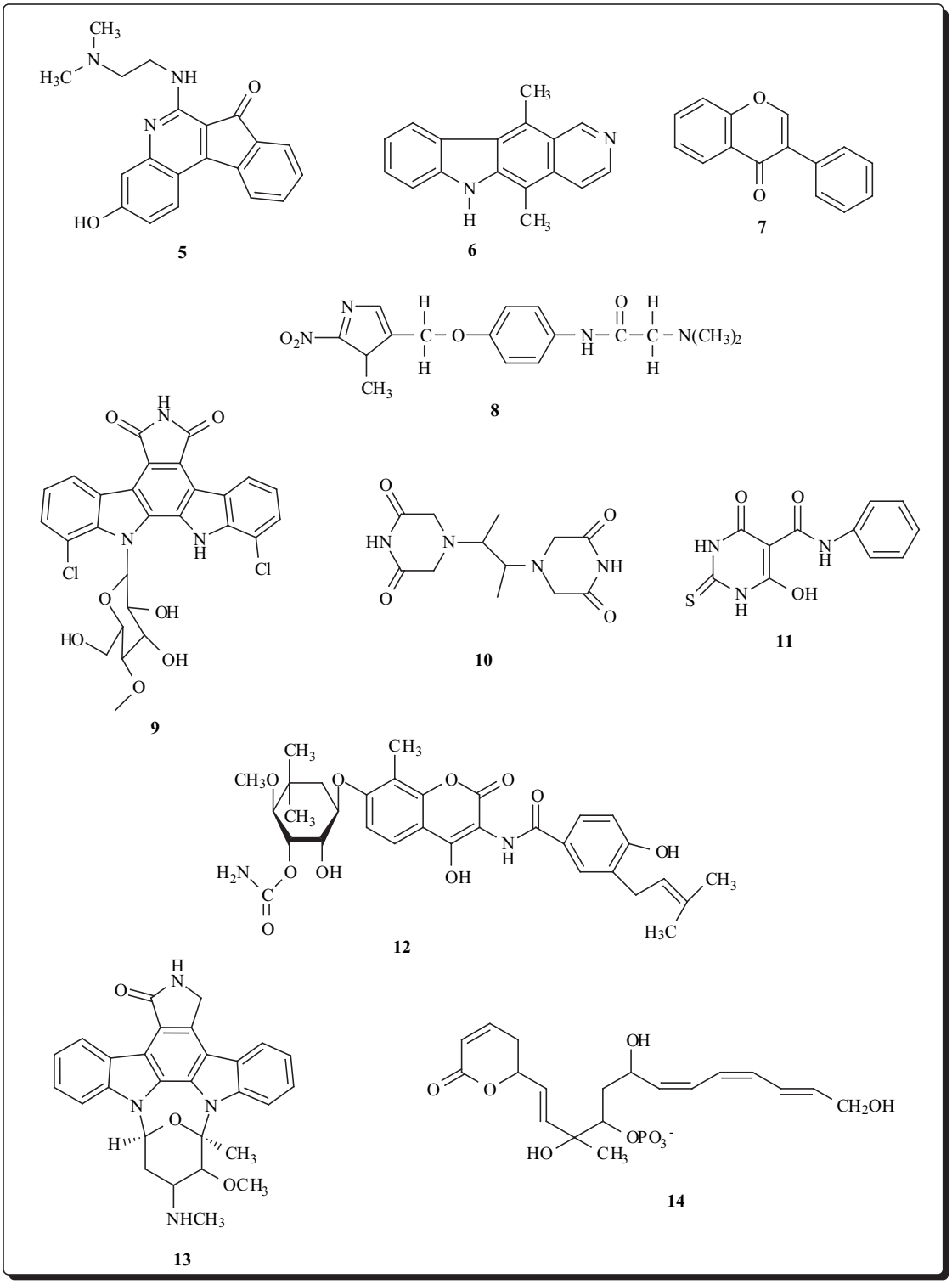
epipodophyllotoxins (*e.g.*, etoposide **3**), and anilinoacridines (**4**); as well as quinolines (**5**), ellipticines (**6**), flavones (**7**), and substituted nitroimidazoles (**8**) [15]. Recent evidence suggests that certain derivatives of rebeccamycin (**9**), an indolocarbazole, may also act as potent poisons [18]. A common structural motif of all these agents is a polycyclic aromatic portion that is believed to interact with DNA [15], perhaps with the transient single-stranded region that occurs within the active site of the enzyme Fig. (**2B**). Most clinical agents (*e.g.*, fluoroquinolones (**1**), the camptothecins (**2**), epipodophyllotoxins (**3**), and some anilinoacridines (*m*-AMSA; **4b**)), increase the half-life of the cleavable complex by inhibiting the religation phase of the reaction. Other compounds (*e.g.,* the ellipticines (**6**), flavones (**7**) and 5-substituted 2-nitroimidazoles (**8**)) achieve the same result by enhancing the rate of DNA cleavage [15]. Evidence has recently been generated to show that bisdioxopiperazines (**10**), which do not have a polycyclic aromatic center and were thought to bind directly to the enzyme, can also act as potent topoisomerase II poisons [19].

Structure-function studies of drug sensitivity and resistance have yielded interesting information on the determinants that govern the intermolecular interactions between enzyme, substrate, and inhibitor. These studies reveal that both the nature and the position of functional groups play an important role in influencing enzyme poisoning. This aspect may be illustrated by studies on the camptothecins, amsacrines and fluoroquinolones. Structure-activity studies on the camptothecins (**2**) have revealed that enzyme-drug interaction is highly stereospecific: the 20-***S*** form is active while the 20-***R*** stereoisomer is not. Additionally, while substitutions on the **A** ring are tolerated, the **E** ring (lactone form) is important and a polarizable group at position 20 is essential [20]. Conclusions derived from these experiments are strongly supported by structure-based modeling studies which reveal that the **E** ring establishes critical contacts with the enzyme within the enzyme-substrate-inhibitor ternary complex [21]. The amsacrines provide a noteworthy example of the influence that seemingly subtle structural features may have on the efficacy of a topoisomerase II poison. While both *m*-AMSA (**4b**) and *o*-AMSA (**4c**) are DNA intercalators (and can inhibit catalysis on that basis), only *m*-AMSA stabilizes cleavable complexes and is a topoisomerase poison [16]. As another illustration, structure-activity analysis of



fluoroquinolones (**1a**) reveals that while the fluorine at C6 is a major determinant of potency, substitutions at the 1, 5, 7 and 8 positions influence the toxicity and specificity of the compounds [22, 23]. Indeed, minor changes in substituents at C7, which preserve antibacterial activity, may profoundly affect potency against mammalian type II topoisomerases [24].

There is substantial evidence to show that determinants on the target protein are also involved in governing enzyme-inhibitor interaction. Studies on camptothecin-resistant topoisomerase I mutants reveal that amino acids flanking the active site play a crucial role in drug binding [25, 26]. Drug interaction is also affected by single amino acid changes in a cluster of residues located at a significant distance from the active site, suggesting the presence of a complex drug interaction domain on the protein [25, 26]. Similar screens for human topoisomerase IIα have revealed that amsacrine binding can be tremendously affected by single amino acid mutations [27]. Competition studies using fluoroquinolones



with other compounds indicate that structurally and mechanistically diverse agents share common or overlapping interaction sites on topoisomerase II [28]. Taken together, these results indicate that structural features of the poison as well as the target protein contribute significantly towards the phenomenon of enzyme poisoning.

Topoisomerase Inhibitors

Aside from the topoisomerase poisons, a large and very diverse assortment of compounds have been reported as topoisomerase inhibitors. These agents have been identified by their ability to inhibit the activity of cell-free enzyme preparations. They may bind directly to the enzyme (*e.g*., ATP analogs) or may bind to and induce structural alterations in the DNA substrate. As expected, these compounds are generally not specific for topoisomerases. Furthermore, when applied to intact cells it is difficult to know whether they inhibit enzyme activity *in situ* or how much (if any) of their killing activity is attributable to topoisomerase inhibition. Of note, because they inhibit topoisomerase-mediated catalysis, these agents can actually suppress the effects of topoisomerase poisons.

Catalytic inhibitors have been identified only for type II enzymes, and they may act at various stages of the catalytic cycle. Agents such as merbarone (**11**) and the coumarins (*e.g.,* novobiocin; **12**) inhibit the DNA cleavage and ATP binding steps of the cycle, respectively [29, 30]. Some topoisomerase-interacting compounds inhibit activity as a coincidental effect of their primary function. Staurosporine (**13**), an indole carbazole and a well-known protein kinase inhibitor, interferes with the ATPase and transesterification reactions of topoisomerase II [31]. Fostriecin (**14**) is a phosphate ester inhibitor of protein phosphatases [32] that was initially characterized as a topoisomerase inhibitor [33].

Agents that bind to DNA may inhibit topoisomerases simply by virtue of their ability to interact with, and induce structural distortion in, the DNA substrate. As a consequence, such compounds may not differentiate between type I and type II topoisomerases in their effects. These include intercalators like aclarubicin and ethidium bromide (**15**) and minor groove DNA binders like distamycin and berenil (**16**) [34-37]. Certain DNA-binding compounds can poison as well as inhibit, depending on their concentration. For example, low levels of *m*-AMSA poison topoisomerase II, but high levels non-specifically inhibit the enzyme due to intercalation and distortion of the DNA substrate [16].

TOPOISOMERASES FROM PROTOZOAN PARASITES

Numerous studies on topoisomerases from parasitic systems have been reported for the kinetoplastids (*e.g*., *Crithidia*, *Trypanosoma*, and *Leishmania* species) and to a lesser extent, the *Plasmodium* species (reviewed in refs. 38-41). This reflects not only the medical importance but also the fascinating biology of these primitive eukaryotes. Particularly relevant to this discussion are two characteristic features of the organisms under consideration: the kinetoplast of *Crithidia*, *Trypanosoma* and *Leishmania* species and the apicoplast of *Plasmodium* species.

The phylogenetic order Kinetoplastida is so named because members contain a kinetoplast, a striking morphological feature visible at the light microscope level. The kinetoplastid parasites include human pathogens (*Trypanosoma, Leishmania*) as well as others that are particularly useful for laboratory studies (*Crithidia*). The kinetoplast is a disc-shaped mass of DNA (termed kinetoplast DNA or kDNA) that is present in the mitochondrion of these organisms. It is comprised of interlocked circular DNA molecules, including thousands of minicircles (1-3 kb each) and dozens of maxicircles (20-40 kb each; see refs. 42-44 for reviews of kDNA structure and function). Maxicircles are the equivalent of conventional mammalian mitochondrial DNA and encode mitochondrial proteins and rRNA. Minicircles come in many different sequence classes and encode guide RNA transcripts that play a role in mRNA processing. This strange structure has no counterpart in mammalian cells. Studies on replication and segregation of the topologically complex kDNA have revealed that topoisomerases play a vital role. Thus kDNA is useful for several purposes: as a component of topoisomerase-directed anti-parasitic therapies; as a valuable reporter system for studying the intracellular function of topoisomerases; and (when purified from cells) as a widely used substrate for assaying the decatenating activity of type II enzymes.

The phylum Apicomplexa contains a number of human pathogens including *Plasmodium* (malaria), *Toxoplasma, Cryptosporidium, Isospora*, and *Babesia*. These parasites all possess a chloroplast-like organelle known as the apicoplast, which carries its own DNA. This is an approximately 35 kb molecule which codes for several species of rRNA and tRNA, and some enzymes involved in protein synthesis [45]. Recent studies have demonstrated that apicoplast function and replication are essential to parasite growth. Its vital function and lack of counterpart in human cells put the apicoplast high on the list of promising drug targets in these important pathogens [46, 47].

Purification and Characterization of Topoisomerases

Type I topoisomerase enzymatic activities have been isolated from *C. fasciculata, T. cruzi*, *L. donovani*, and *P. berghei* [48-51] (Table **2**). As expected from the IB enzymes of higher eukaryotes (Table **1**), all four activities from protozoa reportedly function as monomers, and those from *C. fasciculata*, *T. cruzi* and *P. berghei* have standard catalytic activities. Interestingly, the reported Mg^{2+}-dependence and inability to relax positive supercoils indicates the *L. donovani* enzyme does not follow this pattern and may be prokaryote-like. Topoisomerase I gene sequences are available for *L. donovani* and *P. falciparum* [52, 53]. Curiously, the *Leishmania* gene does not contain the expected catalytic domain motif, although mRNA transcripts can be detected. This raises the possibility that the gene product may have function(s) other than topoisomerase activity, that the catalysis is mediated by another gene product, or perhaps that the catalytic sequence differs from that of higher eukaryotes. Support for the last possibility arises from recent work in which the authors suggest that *Leishmania* topoisomerase I may utilize a serine

Table 2. Topoisomerase I in Protozoan Parasites

Organism	Gene	Native Protein
Crithidia fasciculata	Not reported	79 kDa monomer Does not require Mg^{2+} Not inhibited by ATP Immunolocalizes to nucleus [48]
Trypanosoma cruzi	Not reported	60 kDa monomer Does not require Mg^{2+} Acts on positively and negatively supercoiled DNA [49]
Leishmania donovani	635 aa open reading frame 74 kDa predicated mass Lacks obvious catalytic domain Recombinant protein inactive [52]	67 kDa monomer Requires Mg^{2+} Cannot act on positively supercoiled DNA [50]
Plasmodium falciparum	839 aa open reading frame 92 kDa predicted mass Contains two non-repetitive amino acid insertions [53]	Not reported
Plasmodium berghei	Not reported	70-100 kDa monomer Does not require Mg^{2+} Acts on positively and negatively supercoiled DNA [51]

as the catalytic residue [54], instead of the tyrosine used by every other topoisomerase that has been examined.

Topoisomerase II genes have been cloned from both kinetoplastids and malaria parasites [55-59] (Table **3**). In general, these genes are homologous to their counterparts from higher eukaryotes, although they encode smaller polypeptides. Native enzymes have been isolated from *P. falciparum*, *P. berghei*, *T. cruzi* and *L. donovani* [51, 60-65] (Table **3**). The enzymes from *Plasmodium* species appear to closely resemble eukaryotic type II enzymes in their biochemical behavior. Interestingly, the *T. cruzi* enzyme has been reported to be ATP-independent and ATPase deficient, while there are conflicting reports about the ATP-dependence of the *Leishmania* enzyme. Note that the topoisomerase II genes from both of these kinetoplastids contain ATP-binding domains [57, 58], implying that the observed ATP-independence may be a result of partial proteolysis of the native enzymes during purification, a recognized problem in topoisomerase purification [66, 67]. This possibility is strengthened by the finding that the enzymatic activity of the recombinantly expressed *L. donovani* topoisomerase II is ATP-dependent [58]. However, these data also raise the formal possibility that there exist multiple type II activities in these organisms. Such questions are likely to be settled as the ongoing parasite genome projects approach completion, and the entire complement of parasite topoisomerases is revealed.

Studies on Topoisomerase Expression and Localization

In higher eukaryotes, topoisomerase expression is a complex phenomenon that is regulated at transcriptional,

Table 3. Topoisomerase II in Protozoan Parasites

Organism	Gene	Native Protein
Crithidia fasciculata	1239 aa open reading frame 138 kDa predicted mass [55]	Two activities reported: 132 kDa homodimer [60], and 60 kDa tetramer [61] Both localize to kinetoplast
Trypanosoma brucei	1221 aa open reading frame 137 kDa predicted mass [56]	Not reported
Trypanosoma cruzi	1232 aa open reading frame 138 kDa predicted mass [57]	200 kDa (native) ATPase deficient ATP-independent [63]
Leishmania donovani	1236 aa open reading frame 132 kDa predicted mass Recombinant protein in ATP-dependent [58]	Localizes to nucleus and kinetioplast Conflicting reports on ATP-dependence [64,65]
Plasmadium falciparum	1398 aa open reading frame 160 kDa predicted mass Two asparagine-rich insertions [59]	Partially purified 141kDa plypeptide ATP- and Mg^{2+}-dependent [62]
Plasmodium berghei	Not reported	160 kDa dimer ATP- and Mg^{2+}-dependent [51]

post-transcriptional as well as post-translational level. Comparable studies in protozoans have been few and have focused on kinetoplastids. In these cells regulation apparently does not occur at the transcriptional level, in accordance with the fact that primary transcription of most kinetoplastid genes occurs constitutively and in long polycistronic units [68]. *C. fasciculata* topoisomerase II expression is primarily governed via RNA stability, mediated by a *trans*-acting factor that binds to the 5' untranslated region [69]. In contrast, regulation of *T. cruzi* topoisomerase II apparently occurs at the translational level. Topoisomerase II-specific mRNA is present in equal levels in the replicative and the non-replicative, infective stages. However, the protein is detectable only in the replicative stage [70].

Studies on localization of kinetoplastid topoisomerase I have been restricted to *Crithidia*, wherein the enzyme localizes to the nucleus. Evaluation of topoisomerase II expression and localization has yielded far more intriguing results. Antibodies raised against *C. fasciculata* topoisomerase II localize this enzyme only to the kinetoplast in *C. fasciculata* as well as *T. cruzi* [70, 71]. In contrast, antibodies raised against recombinant *T. cruzi* topoisomerase II react with an exclusively nuclear antigen in both these organisms [70]. This paradox may be due to differences in the epitope preferences of the two antisera. On the other hand, it is also probable that kinetoplastids may possess multiple forms of topoisomerase II. The latter possibility is supported by RNA interference studies on *T. brucei* topoisomerase II where enzyme knockdown results in a selective loss of kDNA, but not nuclear DNA [72].

In *P. falciparum*, expression of both topoisomerase I and II appears to be largely controlled at the level of transcription [73, 74]. However, there is evidence to suggest that *P. falciparum* topoisomerase I may be additionally regulated by post-translational modification [73]. There is no reported information on localization of these enzymes in the parasite.

PROTOZOAN TOPOISOMERASES AS DRUG TARGETS

The utility of the DNA topoisomerases as targets for anti-tumor and anti-bacterial therapies is unequivocally established and multiple lines of evidence suggest they are suitable for anti-parasitic targets. Reported studies include the evaluation of known or putative topoisomerase inhibitors against cell free enzyme preparations, or, alternatively, assessment of the cytotoxicity of such compounds against intact parasites *in vitro*. Few studies demonstrate enzyme inhibition within the cell or provide any evidence to show that cell killing is attributable to (or correlates with) topoisomerase inhibition. The following discussion will focus largely on those compounds that are likely to be specific to the DNA topoisomerases, and for which there is direct evidence of intracellular enzyme inhibition. By definition, this excludes most inhibitors that are not poisons (the coumarins, for example). As for higher eukaryotes, studies with topoisomerase inhibitors not only provide interesting new therapeutic leads, but also afford valuable information on the roles that the DNA topoisomerases play in the nucleic acid metabolism of these pathogens.

Type I Topoisomerases as Targets

Camptothecin (**2a**) is an exquisitely specific inhibitor of topoisomerase I in higher eukaryotes [75], and is a well-characterized antitumor agent [76]. A number of water-soluble camptothecin analogs are currently in clinical use. Camptothecin itself poisons topoisomerase I in African and American trypanosomes, *Leishmania* and *Plasmodium* [77, 78]. In intact cells it promotes the formation of cleavable complexes with both nuclear and mitochondrial DNA, thus providing the first direct evidence for the existence of a mitochondrial type I enzyme. Camptothecin is also cytotoxic to these organisms, and where examined, the magnitude of cytotoxicity correlates directly with cleavable complex formation *in vivo*. Structure-activity analysis reveals that a number of alterations, including most notably the 9-substituted-10, 11-methylenedioxy motif (**2b**) selectively increases potency and specificity for trypanosomes, compared to mammalian cells [79, 80]. These findings provide clear proof of concept that poisoning of topoisomerase I may provide the basis for broad-spectrum antiprotozoal therapy. The latter feature is important, given the limited resources available for antiparasitic drug discovery and development.

Although the camptothecins are the best characterized of the type I topoisomerase inhibitors, several other agents are worth noting. Pentavalent organic antimonials remain the treatment of choice for visceral leishmaniasis. These agents are likely to have a plethora of intracellular targets, however it is of some interest that they promote the formation of cleavable complexes with *Leishmania* topoisomerase I in cell-free preparations and *in situ*, and that these molecular effects correlate well with cytotoxicity [81, 82]. On a more experimental basis, *L. donovani* topoisomerase I can be poisoned by quinone derivatives [83] extracted from plants, suggesting that this chemical class may prove useful in the search for new antileishmanial drugs.

Type II Topoisomerases as Targets

These investigations have largely focused on existing anti-topoisomerase II compounds, with emphasis on the epipodophyllotoxins (**3**), acridine derivatives (**4**), fluoroquinolones (**1a**) and ellipticines (**6**). In addition to practically relevant information, such studies have also yielded significant insights into the functions of these enzymes in nucleic acid metabolism of parasites. For instance, etoposide and the ellipticines poison topoisomerase II in trypanosomes [84, 85]. Etoposide promotes cleavable complex formation with nuclear and mitochondrial DNA indicating the presence of type II topoisomerase in both compartments [85, 86]. Additionally, analysis of the many and diverse lesions seen in kinetoplast DNA from etoposide-treated cells reveals that topoisomerase II plays a structural as well as an essential catalytic role in the mitochondrion [85-87]. Isolated *Plasmodium* topoisomerase II is also sensitive to poisons [51], and cleavable complexes trapped *in vivo* derive from the nucleus as well as the apicoplast [88].

Parasite type II topoisomerases are susceptible to the acridine class of poisons (**4**). Detailed structure-activity

analysis of these compounds has yielded intriguing information on the determinants influencing enzyme-inhibitor interaction. It is known that substitutions on the C9 on the acridine moiety play a critical role: anilino derivatives (**4a**) are effective poisons while their alkylamino counterparts are inactive [89]. Within the subclass of the 9-anilinoacridines (**4a**), the position and nature of substitutions strongly influences specificity towards parasites, as well as the ability of the compound to differentiate between various species of parasites. For instance, substitutions on the acridine ring (primarily at positions 3 and 6) increase potency of these poisons for *Leishmania* and *Plasmodium* as compared to mammalian cells. Differentiation between trypanosomes and *Leishmania* is conferred by substitutions on the aniline moiety [90-93]. These results have established the foundation for further rational modification of these compounds so as to generate potent anti-parasitic agents.

Perhaps the most unexpected and practically useful finding from inhibitor studies has been the demonstration that trypanosomes and *Plasmodium* are sensitive to fluoroquinolones (**1**), generally considered selective for prokaryotic type II topoisomerases [51, 94, 95]. Fluoroquinolones are particularly attractive candidates for anti-parasitic use because of the relative resistance of mammalian topoisomerases. For African trypanosomes, detailed structure-activity analysis revealed that tri-and tetra-cyclic analogs (**1c**) were the most potent and specific, and that cytotoxicity was correlated with topoisomerase II poisoning *in situ* [94]. A variety of fluoroquinolones are active against the malaria parasite *in vitro* [95] and in animal studies [96], and most importantly, have cured humans of uncomplicated malaria [97]. Interestingly, fluoroquinolones stabilize cleavable complexes in the apicoplast, but not the nucleus, of *Plasmodium* [88]. This finding reinforces the notion of multiple pharmacologically distinguishable topoisomerase activities in protozoan parasites.

A number of clinically useful anti-trypanosomal drugs are recognized DNA-binding agents, and their activity against topoisomerase II in parasites is puzzling and incompletely understood. Berenil (**16**) and pentamidine (**17**) are dications that bind in the minor groove of DNA [98], and berenil has long been recognized as an inhibitor, but not a poison, of eukaryotic topoisomerase I [99]. Ethidium bromide (**15**), originally discovered and developed as a treatment for trypanosomiasis, is an intercalating agent with a high affinity for DNA [100]. As an intercalator, it inhibits mammalian topoisomerase activity by distorting the structure of substrate DNA [36]. It was therefore unexpected that each of these agents promotes the formation of double-stranded, protein-bound breaks in minicircle, but not nuclear, DNA indicating that they are *in situ* poisons selective for mitochondrial topoisomerase II [84, 86]. This action underscores the likely existence of more than one type II enzyme in these organisms, and almost certainly explains the propensity of these drugs to generate mutants that have lost their kDNA [101, 102]. Less clear is how much the selective poisoning of mitochondrial topoisomerase activity contributes to the killing of trypanosomes.

De novo screening for topoisomerase II-targeting compounds indicates that quinoline derivatives [103, 104] as well as plant-derived flavonoids may prove to be a valuable source of antiparasitics [105]. In addition to topoisomerase poisoning, these compounds are recognized to have multiple effects on biological systems [106]. Hence, issues of toxicity and specificity will need to be addressed.

SUMMARY

The DNA topoisomerases give every indication of being rewarding targets for antiproliferative strategies directed against protozoan parasites. Inhibitor studies have taught us that parasite topoisomerases tend to be eukaryote-like, especially in their sensitivity to poisons, but that these ancient eukaryotes may harbor prokaryote-like enzymes as well. Structure-activity analyses have revealed subtle but exploitable differences between the parasite enzymes and their mammalian counterparts, thus strengthening the possibility of generating specific agents. In the therapeutic context, perhaps the most important finding has been the demonstration that parasite topoisomerases are sensitive to anti-bacterial fluoroquinolones. Given the relative resistance of mammalian topoisomerases towards these agents, and the myriads of congeners that have been synthesized, fluoroquinolones may well provide the most promising and direct leads for generating potent and specific anti-parasitics.

ACKNOWLEDGEMENTS

Work in the authors' laboratory was supported by the National Institutes of Health (AI28855) and by a Scholar Award in Experimental Therapeutics from the Burroughs Wellcome Fund. We thank Tom Kulikowicz and Jane Scocca for carefully proofing the structures and thoughtfully reading the manuscript, and Dr. Karl Werbovetz for inviting us to prepare this review.

NOTE ADDED IN PROOF

The recent cloning and characterization of native *T. brucei* topoisomerase IB [107] have revealed another remarkable facet of kinetoplastid topoisomerases: the DNA-binding and catalytic functions are divided into two polypeptides encoded by independent genes. Thus the active enzymes is a heteromultimer, in distinct contrast to the type IB enzymes reported from dozens of other organisms. This multimeric structure has been confirmed in *L. donovani* [108] and comparable genes appear in the *T. cruzi* genome database. This unique structural organization provides a promising and novel target for development of specific anti-topoisomerase IB agents.

REFERENCES

[1] Magill, A. J.; Reed, S. G. In *Hunter's Tropical Medicine and Emerging Infectious Diseases*; G. T. Strickland, Ed.; W. B. Saunders Company: Philadelphia, **2000;** pp. 653.

[2] Pepin, J. In *Hunter's Tropical Medicine and Emerging Infectious Diseases*; G. T. Strickland, Ed.; W. B. Saunders Company: Philadelphia, **2000;** pp. 643.

[3] Magill, A. J. In *Hunter's Tropical Medicine and Emerging Infectious Diseases*; G. T. Strickland, Ed.; W. B. Saunders Company: Philadelphia, **2000;** pp. 665.

[4] Taylor, T. E.; Strickland, G. T. In *Hunter's Tropical Medicine and Emerging Infectious Diseases*; G. T. Strickland, Ed.; W. B. Saunders Company: Philadelphia, **2000;** pp. 614.
[5] Maxwell, A.; Gellert, M. *Adv. Protein. Chem.,* **1986,** *38,* 69.
[6] Cozzarelli, N. R. *Science,* **1980,** *207,* 953.
[7] Champoux, J. J. *Annu. Rev. Biochem.,* **2001,** *70,* 369.
[8] Wang, J. C. *Annu. Rev. Biochem.,* **1996,** *65,* 635.
[9] Krogh, B. O.; Shuman, S. *Proc. Natl. Acad. Sci. USA,* **2002,** *99,* 1853.
[10] Belova, G. I.; Prasad, R.; Kozyavkin, S. A.; Lake, J. A.; Wilson, S. H.; Slesarev, A. I. *Proc. Natl. Acad. Sci. USA,* **2001,** *98,* 6015.
[11] Bakshi, R. P.; Galande, S.; Muniyappa, K. *Crit. Rev. Biochem. Mol. Biol.,* **2001,** *36,* 1.
[12] Wang, J. C. *Nat. Rev. Mol. Cell Biol.*, **2002,** *3*, 430.
[13] Zhang, H.; Barcelo, J. M.; Lee, B.; Kohlhagen, G.; Zimonjic, D. B.; Popescu, N. C.; Pommier, Y. *Proc. Natl. Acad. Sci. USA,* **2001,** *98,* 10608.
[14] Liu, L. F. *Annu. Rev. Biochem.,* **1989,** *58,* 351.
[15] Fortune, J. M.; Osheroff, N. *Prog. Nucleic Acid Res. Mol. Biol.,* **2000,** *64,* 221.
[16] Nelson, E. M.; Tewey, K. M.; Liu, L. F. *Proc. Natl. Acad. Sci. USA,* **1984,** *81,* 1361.
[17] Kaufmann, S. H. *Biochim. Biophys. Acta,* **1998,** *1400,* 195.
[18] Woo, M. H.; Vance, J. R.; Marcos, A. R.; Bailly, C.; Bjornsti, M. A. *J. Biol. Chem.,* **2002,** *277,* 3813.
[19] Huang, K. C.; Gao, H.; Yamasaki, E. F.; Grabowski, D. R.; Liu, S.; Shen, L. L.; Chan, K. K.; Ganapathi, R.; Snapka, R. M. *J. Biol. Chem.,* **2001,** *276,* 44488.
[20] Kohn, K. W.; Pommier, Y. *Ann. N. Y. Acad. Sci.,* **2000,** *922,* 11.
[21] Laco, G. S.; Collins, J. R.; Luke, B. T.; Kroth, H.; Sayer, J. M.; Jerina, D. M.; Pommier, Y. *Biochemistry,* **2002,** *41,* 1428.
[22] Gootz, T. D.; Brighty, K. E. In *The Quinolones*; V. T. Andriole, Ed.; Academic Press: San Diego, **1998;** pp. 29.
[23] Domagala, J. M. *J. Antimicrob. Chemother.,* **1994,** *33,* 685.
[24] Gootz, T. D.; McGuirk, P. R.; Moynihan, M. S.; Haskell, S. L. *Antimicrob. Agents Chemother.,* **1994,** *38,* 130.
[25] Champoux, J. J. *Ann. N. Y. Acad. Sci.,* **2000,** *922,* 56.
[26] Larsen, A. K.; Skladanowski, A. *Biochim. Biophys. Acta,* **1998,** *1400,* 257.
[27] Patel, S.; Keller, B. A.; Fisher, L. M. *Mol. Pharmacol.,* **2000,** *57,* 784.
[28] Corbett, A. H.; Hong, D.; Osheroff, N. *J. Biol. Chem.,* **1993,** *268,* 14394.
[29] Fortune, J. M.; Osheroff, N. *J. Biol. Chem.,* **1998,** *273,* 17643.
[30] Kreuzer, K. N.; Cozzarelli, N. R. *Cell,* **1980,** *20,* 245.
[31] Lassota, P.; Singh, G.; Kramer, R. *J. Biol. Chem.,* **1996,** *271,* 26418.
[32] Roberge, M.; Tudan, C.; Hung, S. M.; Harder, K. W.; Jirik, F. R.; Anderson, H. *Cancer Res.,* **1994,** *54,* 6115.
[33] Boritzki, T. J.; Wolfard, T. S.; Besserer, J. A.; Jackson, R. C.; Fry, D. W. *Biochem. Pharmacol.,* **1988,** *37,* 4063.
[34] Pilch, D. S.; Kirolos, M. A.; Liu, X.; Plum, G. E.; Breslauer, K. J. *Biochemistry,* **1995,** *34,* 9962.
[35] Woynarowski, J. M.; McHugh, M.; Sigmund, R. D.; Beerman, T. A. *Mol. Pharmacol.,* **1989,** *35,* 177.
[36] Pommier, Y.; Covey, J. M.; Kerrigan, D.; Markovits, J.; Pham, R. *Nucleic Acids Res.,* **1987,** *15,* 6713.
[37] Sorensen, B. S.; Sinding, J.; Andersen, A. H.; Alsner, J.; Jensen, P. B.; Westergaard, O. *J. Mol. Biol.,* **1992,** *228,* 778.
[38] Cheesman, S. J. *Parasitol. Today,* **2000,** *16,* 277.
[39] Nenortas, E. C.; Bodley, A. L.; Shapiro, T. A. *Biochim. Biophys. Acta,* **1998,** *1400,* 349.
[40] Burri, C.; Bodley, A. L.; Shapiro, T. A. *Parasitol. Today,* **1996,** *12,* 226.
[41] Shapiro, T. A. *Adv. Pharmacol.,* **1994,** *29B,* 187.
[42] Simpson, L. *Int. Rev. Cytol.,* **1986,** *99,* 119.
[43] Shapiro, T. A.; Englund, P. T. *Annu. Rev. Microbiol.,* **1995,** *49,* 117.
[44] Morris, J. C.; Drew, M. E.; Klingbeil, M. M.; Motyka, S. A.; Saxowsky, T. T.; Wang, Z.; Englund, P. T. *Int. J. Parasitol.,* **2001,** *31,* 453.
[45] Wilson, R. J.; Denny, P. W.; Preiser, P. R.; Rangachari, K.; Roberts, K.; Roy, A.; Whyte, A.; Strath, M.; Moore, D. J.; Moore, P. W.; Williamson, D. H. *J. Mol. Biol.,* **1996,** *261,* 155.
[46] Soldati, D. *Parasitol. Today,* **1999,** *15,* 5.
[47] McFadden, G. I.; Roos, D. S. *Trends Microbiol.,* **1999,** *7,* 328.
[48] Melendy, T.; Ray, D. S. *Mol. Biochem. Parasitol.,* **1987,** *24,* 215.
[49] Riou, G. F.; Gabillot, M.; Douc-Rasy, S.; Kayser, A.; Barrois, M. *Eur. J. Biochem.,* **1983,** *134,* 479.
[50] Chakraborty, A. K.; Gupta, A.; Majumder, H. K. *Indian J. Biochem. Biophys.,* **1993,** *30,* 257.
[51] Riou, J. F.; Gabillot, M.; Philippe, M.; Schrevel, J.; Riou, G. *Biochemistry,* **1986,** *25,* 1471.
[52] Broccoli, S.; Marquis, J. F.; Papadopoulou, B.; Olivier, M.; Drolet, M. *Nucleic Acids Res.,* **1999,** *27,* 2745.
[53] Tosh, K.; Kilbey, B. *Gene,* **1995,** *163,* 151.
[54] Das, A.; Mandal, C.; Dasgupta, A.; Sengupta, T.; Majumder, H. K. *Nucleic Acids Res.,* **2002,** *30,* 794.
[55] Pasion, S. G.; Hines, J. C.; Aebersold, R.; Ray, D. S. *Mol. Biochem. Parasitol.,* **1992,** *50,* 57.
[56] Strauss, P. R.; Wang, J. C. *Mol. Biochem. Parasitol.,* **1990,** *38,* 141.
[57] Fragoso, S. P.; Goldenberg, S. *Mol. Biochem. Parasitol.,* **1992,** *55,* 127.
[58] Das, A.; Dasgupta, A.; Sharma, S.; Ghosh, M.; Sengupta, T.; Bandopadhyay, S.; Majumder, H. K. *Nucleic Acids Res.,* **2001,** *29,* 1844.
[59] Cheesman, S.; McAleese, S.; Goman, M.; Johnson, D.; Horrocks, P.; Ridley, R. G.; Kilbey, B. J. *Nucleic Acids Res.,* **1994,** *22,* 2547.
[60] Melendy, T.; Ray, D. S. *J. Biol. Chem.,* **1989,** *264,* 1870.
[61] Shlomai, J.; Zadok, A.; Frank, D. *Adv. Exp. Med. Biol.,* **1984,** *179,* 409.
[62] Chavalitshewinkoon, P.; Leelaphiwat, S.; Wilairat, P. *Southeast Asian J. Trop. Med. Public Health,* **1994,** *25,* 32.
[63] Douc-Rasy, S.; Kayser, A.; Riou, J. F.; Riou, G. *Proc. Natl. Acad. Sci. USA,* **1986,** *83,* 7152.
[64] Chakraborty, A. K.; Majumder, H. K. *Mol. Biochem. Parasitol.,* **1987,** *26,* 215.
[65] Chakraborty, A. K.; Majumder, H. K. *Biochem. Biophys. Res. Commun.,* **1991,** *180,* 279.
[66] Sander, M.; Hsieh, T. *J. Biol. Chem.,* **1983,** *258,* 8421.
[67] Schmitt, B.; Buhre, U.; Vosberg, H. P. *Eur. J. Biochem.,* **1984,** *144,* 127.
[68] Vanhamme, L.; Pays, E. *Microbiol. Rev.,* **1995,** *59,* 223.
[69] Mahmood, R.; Hines, J. C.; Ray, D. S. *Mol. Cell Biol.,* **1999,** *19,* 6174.
[70] Fragoso, S. P.; Mattei, D.; Hines, J. C.; Ray, D.; Goldenberg, S. *Mol. Biochem. Parasitol.,* **1998,** *94,* 197.
[71] Melendy, T.; Sheline, C.; Ray, D. S. *Cell,* **1988,** *55,* 1083.
[72] Wang, Z.; Morris, J. C.; Drew, M. E.; Englund, P. T. *J. Biol. Chem.,* **2000,** *275,* 40174.
[73] Tosh, K.; Cheesman, S.; Horrocks, P.; Kilbey, B. *Exp. Parasitol.,* **1999,** *91,* 126.
[74] Cheesman, S.; Horrocks, P.; Tosh, K.; Kilbey, B. *Mol. Biochem. Parasitol.,* **1998,** *92,* 39.
[75] Nitiss, J.; Wang, J. C. *Proc. Natl. Acad. Sci. USA,* **1988,** *85,* 7501.
[76] Muggia, F. M.; Dimery, I.; Arbuck, S. G. *Ann. N. Y. Acad. Sci.,* **1996,** *803,* 213.
[77] Bodley, A. L.; Cumming, J. N.; Shapiro, T. A. *Biochem. Pharmacol.,* **1998,** *55,* 709.
[78] Bodley, A. L.; Shapiro, T. A. *Proc. Natl. Acad. Sci. USA,* **1995,** *92,* 3726.
[79] Bodley, A. L.; Wani, M. C.; Wall, M. E.; Shapiro, T. A. *Biochem. Pharmacol.,* **1995,** *50,* 937.
[80] Werbovetz, K. A.; Bhattacharjee, A. K.; Brendle, J. J.; Scovill, J. P. *Bioorg. Med. Chem.,* **2000,** *8,* 1741.
[81] Chakraborty, A. K.; Majumder, H. K. *Biochem. Biophys. Res. Commun.,* **1988,** *152,* 605.
[82] Lucumi, A.; Robledo, S.; Gama, V.; Saravia, N. G. *Antimicrob. Agents Chemother.,* **1998,** *42,* 1990.
[83] Ray, S.; Hazra, B.; Mittra, B.; Das, A.; Majumder, H. K. *Mol. Pharmacol.,* **1998,** *54,* 994.
[84] Shapiro, T. A.; Englund, P. T. *Proc. Natl. Acad. Sci. USA,* **1990,** *87,* 950.
[85] Shapiro, T. A.; Klein, V. A.; Englund, P. T. *J. Biol. Chem.,* **1989,** *264,* 4173.
[86] Shapiro, T. A. *Acta Trop.,* **1993,** *54,* 251.
[87] Shapiro, T. A.; Showalter, A. F. *Mol. Cell Biol.,* **1994,** *14,* 5891.
[88] Weissig, V.; Vetro-Widenhouse, T. S.; Rowe, T. C. *DNA Cell Biol.,* **1997,** *16,* 1483.
[89] Werbovetz, K. A.; Spoors, P. G.; Pearson, R. D.; Macdonald, T. L. *Mol. Biochem. Parasitol.,* **1994,** *65,* 1.
[90] Figgitt, D.; Denny, W.; Chavalitshewinkoon, P.; Wilairat, P.; Ralph, R. *Antimicrob. Agents Chemother.,* **1992,** *36,* 1644.

[91] Gamage, S. A.; Figgitt, D. P.; Wojcik, S. J.; Ralph, R. K.; Ransijn, A.; Mauel, J.; Yardley, V.; Snowdon, D.; Croft, S. L.; Denny, W. A. *J. Med. Chem.,* **1997,** *40,* 2634.
[92] Chavalitshewinkoon, P.; Wilairat, P.; Gamage, S.; Denny, W.; Figgitt, D.; Ralph, R. *Antimicrob. Agents Chemother.,* **1993,** *37,* 403.
[93] Gamage, S. A.; Tepsiri, N.; Wilairat, P.; Wojcik, S. J.; Figgitt, D. P.; Ralph, R. K.; Denny, W. A. *J. Med. Chem.,* **1994,** *37,* 1486.
[94] Nenortas, E.; Burri, C.; Shapiro, T. A. *Antimicrob. Agents Chemother.,* **1999,** *43,* 2066.
[95] Divo, A. A.; Sartorelli, A. C.; Patton, C. L.; Bia, F. J. *Antimicrob. Agents Chemother.,* **1988,** *32,* 1182.
[96] Salmon, D.; Deloron, P.; Gaudin, C.; Malhotra, K.; Lebras, J.; Pocidalo, J. J. *Antimicrob. Agents Chemother.,* **1990,** *34,* 2327.
[97] Sarma, P. S. *Ann. Intern. Med.,* **1989,** *111,* 336.
[98] Reinert, K. E. *J. Biomol. Struct. Dyn.,* **1999,** *17,* 311.
[99] Fairfield, F. R.; Bauer, W. R.; Simpson, M. V. *Biochim. Biophys. Acta,* **1985,** *824,* 45.
[100] Neidle, S.; Abraham, Z. *CRC Crit. Rev. Biochem.,* **1984,** *17,* 73.
[101] Riou, G.; Benard, J. *Biochem. Biophys. Res. Commun.,* **1980,** *96,* 350.
[102] Riou, G. F.; Belnat, P.; Benard, J. *J. Biol. Chem.,* **1980,** *255,* 5141.
[103] Ray, S.; Sadhukhan, P. K.; Mandal, N. B.; Mahato, S. B.; Majumder, H. K. *Biochem. Biophys. Res. Commun.,* **1997,** *230,* 171.
[104] Slunt, K. M.; Grace, J. M.; Macdonald, T. L.; Pearson, R. D. *Antimicrob. Agents Chemother.,* **1996,** *40,* 706.
[105] Mittra, B.; Saha, A.; Chowdhury, A. R.; Pal, C.; Mandal, S.; Mukhopadhyay, S.; Bandyopadhyay, S.; Majumder, H. K. *Mol. Med.,* **2000,** *6,* 527.
[106] Hodek, P.; Trefil, P.; Stiborova, M. *Chem. Biol. Interact.,* **2002,** *139,* 1.
[107] Bodley, A. L.; Chakraborty, A. K.; Xie, S.; Burri, C.; Shapiro, T. A. *Proc. Natl. Acad. Sci. USA*, **2003**, in press.
[108] Villa, H.; Marcos, A. R. O.; Reguera, R. M.; Balana-Fouce, R.; Garcia-Estrada, C.; Perez-Pertejo, Y.; Tekwani, B. L.; Myler, P. J.; Stuart, K. D.; Bjornsti, M. A.; Ordonez, D. *J. Biol. Chem.*, **2003**, *278*, 3521.