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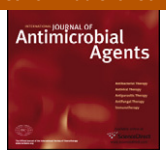

Effect of topoisomerase inhibitors and DNA-binding drugs on the cell proliferation and ultrastructure of *Trypanosoma cruzi*

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ABSTRACT

Trypanosomatids present unusual organelles, such as the kinetoplast that contains the mitochondrial DNA arranged in catenated circles. The nucleus of these protozoa presents distinct domains during interphase as well as a closed mitosis. DNA topoisomerases modulate the topological state of DNA by regulating supercoiling of the double-stranded DNA during replication, transcription, recombination and repair. Because topoisomerases play essential roles in cellular processes, they constitute a potential target for antitumour and antimicrobial drugs. In this study, the effects of various topoisomerase inhibitors and DNA-binding drugs were tested on the cellular proliferation and ultrastructure of the *Trypanosoma cruzi* epimastigote form *Blastocrithidia culicis* was used as a comparative model, which has a more relaxed kinetoplast DNA (kDNA) organization. The results showed that the eukaryotic topoisomerase I inhibitors camptothecin and rebeccamycin were the most effective compounds in the arrest of *T. cruzi* proliferation. Of the eukaryotic topoisomerase II inhibitors, mitoxantrone, but not merbarone, was effective against cell proliferation. The prokaryotic topoisomerase II inhibitors norfloxacin and enoxacin targeted the kinetoplast specifically, thus promoting ultrastructural kDNA rearrangement in *B. culicis*. Of the DNA-binding drugs, berenil caused remarkable kDNA disorganization. With the exception of camptothecin, there have been no previous evaluations of the compounds tested here on trypanosomatid ultrastructure. In conclusion, inhibitors of the same class may have different effects on trypanosomatid proliferation and ultrastructure. The results obtained in this work may help to reveal the mechanism of action of different topoisomerase inhibitors in trypanosomatids.

1. Introduction

DNA topoisomerases are enzymes that regulate the DNA topological state by introducing or removing supercoiling, knots or catenations in DNA molecules. The dynamic nature of DNA is required for its essential biological functions such as replication, transcription, recombination, repair and DNA segregation [1–3]. Type I enzymes modify DNA topology by a single-stranded DNA passage mechanism and are usually referred to as topoisomerase I (topo I). Type II enzymes (topoisomerase II, or topo II), which includes eukaryotic topo II and the bacterial DNA gyrases, hydrolyse ATP and alter topology by a double-stranded DNA passage mechanism.

The family Trypanosomatidae includes protozoa of medical importance such as *Leishmania* spp., *Trypanosoma brucei* and *Trypanosoma cruzi*, the latter being the aetiological agent of Chagas disease. These protozoan cells contain a single nucleus that presents distinct domains during interphase and maintains its envelope integrity during closed mitosis, and the chromosomes are not observed [4–7]. The single mitochondrion presents an enlarged portion containing its DNA, the kinetoplast, which is composed of interlocked minicircles and maxicircles that form a network with a unique arrangement in nature. During replication of kinetoplast DNA (kDNA), the covalently closed minicircles are released from the network by topo II enzymes. Following replication, the free circles migrate to the antipodal sites, which correspond to enzyme complexes that are located 180° apart. Finally, minicircles are reattached to the kDNA disk and are distributed through the network, before kinetoplast scission [8–10].

Topoisomerases I and II have been characterized in several trypanosomatids [11–19]. Topo I activity is ATP-independent and the enzyme is composed of two subunits encoded by two different genes, with the C-terminal catalytic domain being highly conserved [17]. Topo II has ATP-dependent and -independent decatenating

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activities and presents separate nuclear and mitochondrial-encoding genes [20,21]. With regard to cellular localization, such enzymes are found in the nucleus and in the kinetoplast of trypanosomatid protozoa [22].

Agents that affect DNA topoisomerases have proven to be useful for cancer treatment, but there have been less extensive studies on trypanosomatids. Most topo I-targeted drugs act by stabilizing the covalent topoisomerase–DNA complex, thus preventing re-ligation. As a consequence, topo I inhibitors exhibit cell cycle arrest with activation of DNA damage signals that induce DNA repair mechanisms or apoptosis [23–26]. Topo II inhibitors such as nalidixic acid, etoposide and mitoxantrone enhance irreversible topo II-mediated DNA cleavages, thus transforming this essential enzyme into a potent cellular toxin. Conversely, topo II inhibitors, which affect enzyme activity, do not have the ability to stimulate DNA cleavage like novobiocin, aclarubicin, merbarone and staurosporine [27–29]. DNA-binding drugs such as berenil, pentamidine and related diamidine analogues are catalytic inhibitors that may prevent the binding of topoisomerase to DNA, stabilize non-covalent DNA–topoisomerase complexes or prevent the binding of ATP to the enzyme [30–32]. In trypanosomatids, a few reports have proposed that such groove-binder drugs interact preferentially with AT-rich mitochondrial DNA rather than with nuclear DNA. This is in agreement with the proposal that topo II is unable to interact with DNA sites where the minor groove is occupied by ligand drugs [33–35].

In this study, the effects of the eukaryotic topo I inhibitors camptothecin and rebeccamycin, the eukaryotic topo II inhibitors mitoxantrone and merbarone, the prokaryotic topo II (gyrase) inhibitors norfloxacin and enoxacin, and the groove-binder compounds berenil and distamycin were tested in the proliferative epimastigote form of *T. cruzi* with regard to protozoan proliferation and ultrastructure. In assays using prokaryotic topo II inhibitors and DNA-binding drugs, which mainly target the kinetoplast, the monoxenic trypanosomatid *Blastocrithidia culicis* was used as a comparative model as it presents a looser kDNA arrangement that is usually more sensitive to drugs that target the kinetoplast.

2. Materials and methods

2.1. Protozoa culture

Epimastigote forms of *T. cruzi* and *B. culicis* were grown for 24 h at 28 °C in liver infusion tryptose [36] or Warren medium [37], respectively, supplemented with 10% fetal calf serum.

2.2. Chemicals

Rebeccamycin, camptothecin and mitoxantrone, as well as merbarone, were diluted in dimethyl sulphoxide (DMSO) and were stored at concentrations of 0.1, 5 and 10 mM, respectively. Berenil (diminazene aceturate) and distamycin were dissolved in water at 5 mM and 1 mM, respectively. Norfloxacin and enoxacin were diluted in 100 mM NaOH solution. All drugs were purchased from Sigma-Aldrich (São Paulo, Brazil) and were stored at 4 °C.

2.3. Drug treatment

Cells were grown as previously described and drugs were added to the culture medium after 24 h of growth. Drug concentrations for treatment were determined according to data presented in previous reports [28,34,38–40] and were used as follows: camptothecin, 1, 5, 10 and 50 μM; rebeccamycin, 1, 5 and 10 μM; merbarone, 50, 100, 200 and 300 μM; mitoxantrone, 5, 10, 20 and 50 μM; norfloxacin and enoxacin, 156, 470, 940 and 1560 μM; berenil, 2, 10, 20 and 50 μM; and distamycin, 5, 10, 20, 40 and 100 μM. During 96 h of cell growth, trypanosomatids were collected every 24 h for cell counting in a Neubauer chamber or for transmission electron microscopy (TEM). To compare control and treated groups, paired *t*-tests were applied to results using the 95% confidence interval (GraphPad Prism version 5.00 for windows; GraphPad Software Inc., San Diego, CA).

To determine cell viability, control and treated cells were incubated with 0.1% trypan blue diluted in distilled water for 5 min. After this period, live protozoa remained uncoloured, while dead cells were permeable to the blue solution and thus stained blue.

2.4. Transmission electron microscopy

Protozoa were fixed in 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer (pH 7.2) at room temperature and were washed in the same buffer. Cells were post-fixed in 0.1 M cacodylate buffer containing 1% O_sO_4 and 0.8% potassium ferricyanide for 1 h. Then, protozoa were washed in the same buffer and were dehydrated in a graded series of acetone and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and were observed using a Zeiss 900 transmission electron microscope (Zeiss, Oberkochen, Germany).

3. Results

Cell growth of *T. cruzi* epimastigote forms was highly affected after treatment with camptothecin, resulting in the lowest 50% inhibitory concentration (IC_{50}) value (2.08 μM) of all tested inhibitors. This compound promoted a dose-dependent inhibitory effect that induced cell proliferation arrest after treatment with 5, 10 and 50 μM for 48 h (Fig. 1a). Rebeccamycin also induced significant growth inhibition, but its IC_{50} value (6.04 μM) was higher than that for camptothecin. Rebeccamycin did not inhibit cell proliferation at its lowest concentration (1 μM) and growth impairment was only observed after treatment of protozoa with 10 μM for

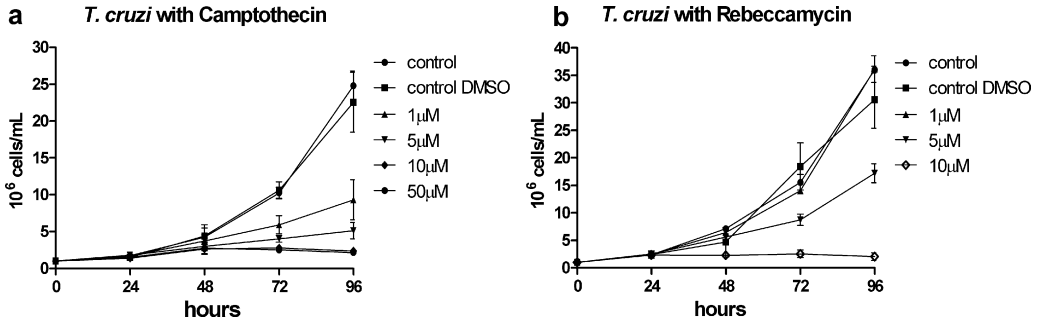

Fig. 1. Growth inhibition of *Trypanosoma cruzi* epimastigote form following treatment with the eukaryote topoisomerase I inhibitors (a) camptothecin and (b) rebeccamycin. Data are the average of three independent experiments. DMSO, dimethyl sulphoxide.

24 h (Fig. 1b). Ultrastructural features of the cells were analysed by TEM. The condensed chromatin, which is usually found close to the nuclear envelope and around the nucleolus (Fig. 2a) was unpacked, revealing round and electron-dense structures (Fig. 2b and c, arrows). Such alterations were observed following cell growth in the presence of 10 μM of camptothecin and rebeccamycin for 48 h. Higher drug concentrations, such as 50 μM, induced mitochondrial swelling (Fig. 2b and c, asterisk), but the kinetoplast ultrastructure was not affected.

Merbarone did not promote significant growth inhibition in *T. cruzi* epimastigotes. Cellular proliferation was not affected following treatment with 100 μM for 72 h, and treatment with a higher drug concentration (300 μM) decreased protozoa growth by only 50% (Fig. 3a). Conversely, mitoxantrone induced a strong inhibitory effect on cellular proliferation (IC_{50} = 5.37 μM) that was similar to that obtained following treatment with camptothecin and rebeccamycin. Cell proliferation was inhibited after treatment with 5 μM for 24 h and growth arrest was observed when higher doses (10–50 μM) were applied for 48 h (Fig. 3b). Merbarone- and mitoxantrone-treated cells exhibited cristae swelling in the mitochondrion following drug treatment (Fig. 4a). Curiously, mitoxantrone promoted the loss of reservosome content (Fig. 4b) following treatment with higher doses (20–50 μM) for 72 h.

Norfloxacin had a dose-dependent inhibitory effect on cell proliferation of *T. cruzi* epimastigotes. However, inhibition of cell proliferation was only observed after drug treatment with 156 μM for 48 h (Fig. 5a). Norfloxacin was also tested in *B. culicis*, a species that has been used as a comparative model for presenting a looser kDNA network that is usually more sensitive to drugs targeting the kinetoplast [39,41]. The results showed that cell proliferation of *B. culicis* was more affected by norfloxacin than that of *T. cruzi*. *Blastocrithidia culicis* exhibited cell growth arrest after treatment with 156 μM norfloxacin for 48 h (Fig. 5b). These data gave an IC_{50} value that is five-fold lower in *B. culicis* (IC_{50} < 156 μM). Enoxacin was more effective in blocking cell proliferation of both species (Fig. 5c and d). In *T. cruzi*, the number of parasites was 50% lower compared with control cells after treatment with 156 μM of drug for 48 h (Fig. 5c). This result was only observed in protozoa treated with norfloxacin at 974 μM (the IC_{50}) for 48 h (Fig. 5a). *Blastocrithidia culicis* proliferation was also more sensitive to enoxacin, as cell growth impairment was observed in trypanosomatids treated with 156 μM of drug for 48 h (Fig. 5d). Norfloxacin induced significant cytoplasmic vacuolation in *T. cruzi* (Fig. 6a), and enoxacin-treated cells exhibited an enlarged space between the inner and outer membranes that enclose the nucleus following treatment with 156 μM for 48 h (Fig. 6b). *Trypanosoma cruzi* did not show ultrastructural modifications either in kDNA or nuclear DNA following treatment with norfloxacin or enoxacin (Fig. 6a and b). However, in *B. culicis* the kDNA showed compaction after treatment with norfloxacin and enoxacin. These inhibitors promoted the formation of thicker DNA fibre strands as well as an electron-dense band at the centre of the kinetoplast network. Such ultrastructural effects were more intense following norfloxacin treatment (Fig. 6d and e).

Berenil is a DNA minor groove-binding compound and is considered a topoisomerase-targeting agent [42] that mainly affects mitochondrial topo II [33]. This compound was effective against *T. cruzi* proliferation, as growth inhibition was observed following treatment with 20 μM for 24 h (Fig. 7a). Curiously, *B. culicis* cell proliferation was less affected by berenil treatment than *T. cruzi* epimastigotes (Fig. 7b). As such, the IC_{50} value was more than two-fold higher in *B. culicis* (46.73 μM) than in *T. cruzi* (20.25 μM). In contrast, distamycin, an aromatic diamidine, was a less potent inhibitor of *T. cruzi*, with an IC_{50} value (98.58 μM) that was five-fold higher than the value obtained for berenil (Fig. 7c). The effect of distamycin on *B. culicis* proliferation was similar to that described for *T. cruzi*,

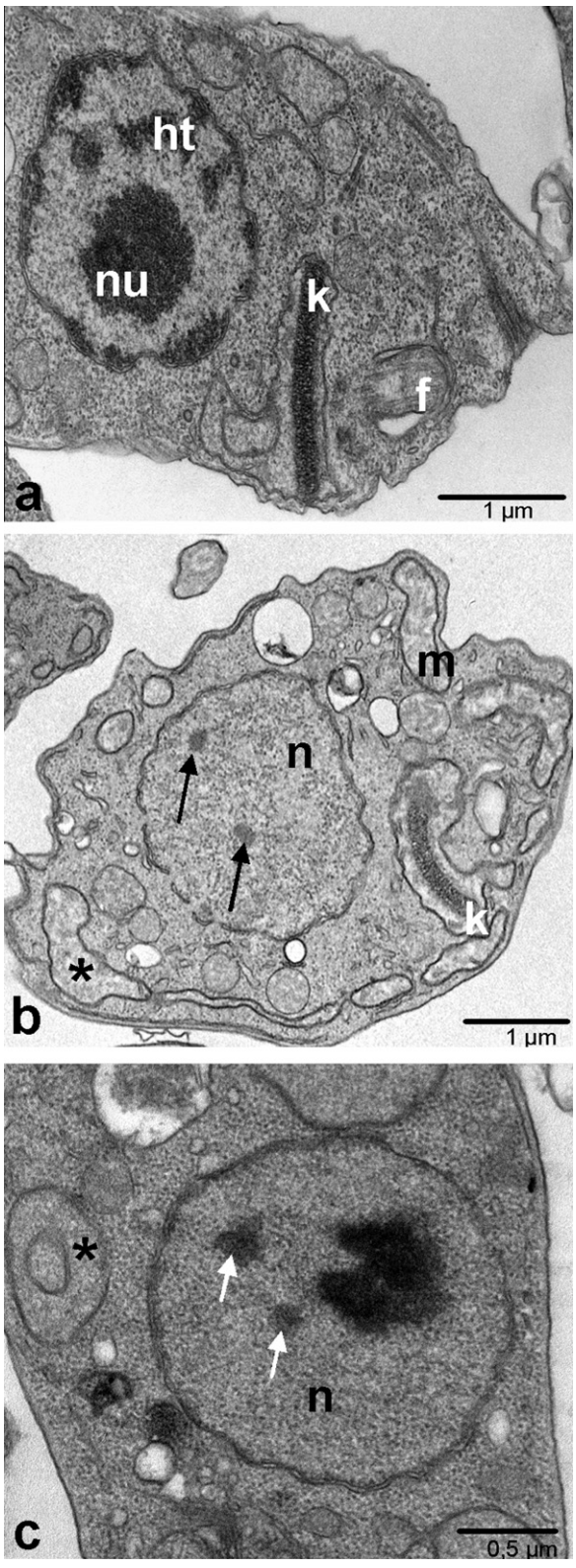

Fig. 2. (a) Non-treated epimastigote form of *Trypanosoma cruzi* showing the nucleus (n) with the peripherally condensed heterochromatin (ht), nucleolus (nu), kinetoplast (k) and flagellum (f). (b and c) *T. cruzi* treated for 48 h with 10 μM of camptothecin (b) or rebeccamycin (c). Note the unpacked heterochromatin revealing electron-dense structures, which could correspond to Cajal bodies (arrows), and the mitochondrion (m) swelling (*).

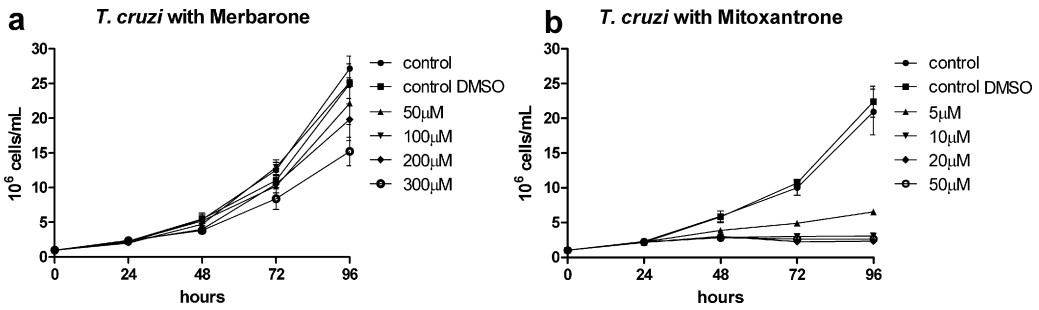

Fig. 3. Growth inhibition of *Trypanosoma cruzi* epimastigote form following treatment with the eukaryote topoisomerase II inhibitors (a) merbarone and (b) mitoxantrone. Data are the average of three independent experiments. DMSO, dimethyl sulphoxide.

with an IC_{50} value of 101.11 μM (Fig. 7d). The most striking effect of berenil treatment was observed on the kinetoplast. In treated epimastigotes of *T. cruzi*, mitochondria exhibited loss of the matrix and swelling after treatment with 2 μM for 48 h. Some cells also displayed an electron-dense point close to the kinetoplast antipodal sites (Fig. 8a). When the drug concentration was increased to 10, 20 and 50 μM, the most remarkable effect observed after 48 h was modification of the kDNA arrangement; many cells displayed a kinetoplast disk with electron-lucid areas (Fig. 8b) and with membrane profiles in the middle of the kDNA region (Fig. 8c), corresponding to invaginations of the inner mitochondrial membrane (Fig. 8d). Such kinetoplast alterations have never been described before. Regarding the ultrastructure of *B. culicis*, cells treated with berenil or distamycin also presented kDNA rearrangement. However, these alterations were similar to those described following treatment with norfloxacin and enoxacin, since thicker DNA fibre strands were observed corresponding to a higher kDNA compaction compared with control cells (Fig. 6e).

It is worth mentioning that the viability test with trypan blue showed that 100% of cells were viable when *T. cruzi* or *B. culicis* were treated with the highest concentration of all evaluated compounds for 72 h (data not shown).

Table 1 summarises the effects of topoisomerase inhibitors and DNA-binding drugs evaluated in this study.

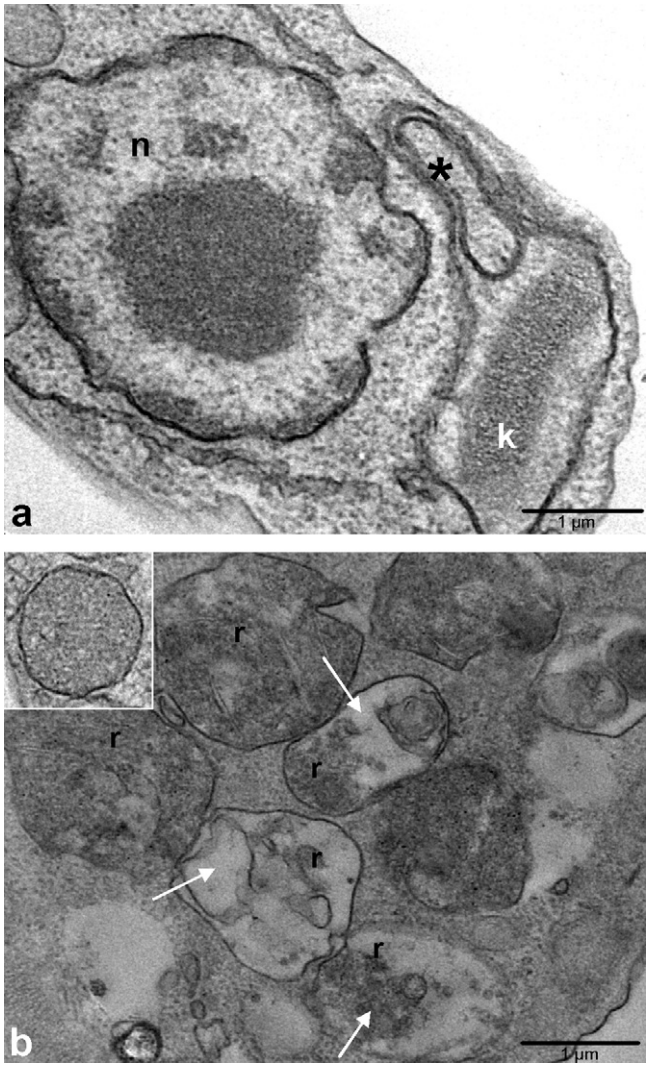

Fig. 4. (a) *Trypanosoma cruzi* treated with 300 μM merbarone for 72 h. No ultrastructural changes were observed in the nucleus (n) or kinetoplast (k), but some protozoa exhibited cristae swelling (*). (b) *T. cruzi* treated with 20 μM mitoxantrone for 72 h presented loss of reservosome (r) content (arrows). Inset shows a reservosome of a non-treated cell.

4. Discussion

DNA topoisomerases were recognized as promising targets in cancer therapy in the late 1980s [43], considering its pivotal role in essential biological process. As in most eukaryotic cells, topoisomerases are essential enzymes in trypanosomatids, as they are involved in nuclear and mitochondrial DNA metabolism. RNA interference (RNAi)-mediated silencing of each subunit of *T. brucei* topo IB results in a drastic reduction both of DNA and RNA synthesis, as well as inhibition of parasite growth [44]. The essential role of topo II for kinetoplast maintenance and replication was demonstrated in *T. brucei* topo II RNAi cells that present a progressive shrinkage and loss of kDNA [45]. Trypanosomatid DNA topoisomerases are distinct from human counterparts with regard to their biological properties and preferential sensitivity to several compounds, justifying the use of these enzymes as cellular targets in chemotherapy against trypanosomiasis [22,46–48].

Type IB topoisomerases are the sole molecular target of camptothecin, the best-characterized topo I inhibitor and an important antitumour agent [49,50]. Camptothecin is an uncompetitive inhibitor that binds to the covalent intermediate DNA–enzyme, thereby stabilizing the cleavable complex and reducing re-ligation. During DNA duplication, an advancing replication fork collides with the inhibitor-trapped topo I cleavable complex, generating DNA double-stranded breaks that interact with repair enzymes, which activates checkpoint kinases. Thus, cells either arrest in the G2-M cell cycle for DNA repair or proceed to apoptosis [26,51,52]. In previous work with African trypanosomes, camptothecin promoted covalent protein–DNA complex formation both with nuclear and kinetoplast DNA and inhibited DNA biosynthesis. Furthermore, the drug was cytotoxic to *T. brucei*, *T. cruzi* and *Leishmania donovani*, with IC_{50} values ranging from 1 μM to 3 μM [38]. In the present work, camptothecin was the most potent inhibitor of *T. cruzi* cell

Table 1
Effects of different topoisomerase inhibitors and DNA-binding drugs in *Trypanosoma cruzi* and *Blastocrithidia culicis*.

Drug	Target	Species in which the compound was tested	IC_{50} for *T. cruzi* (μM)	Effects
Camptothecin	Eukaryotic topo I inhibitor	*T. cruzi*	2.08	Unpacking of heterochromatin and mitochondrial swelling
Rebeccamycin	Eukaryotic topo I inhibitor	*T. cruzi*	6.04	Unpacking of heterochromatin and mitochondrial swelling
Merbarone	Eukaryotic topo II inhibitor	*T. cruzi*	338.17	Mitochondrial swelling
Mitoxantrone	Eukaryotic topo II inhibitor	*T. cruzi*	5.37	Loss of reservosome content
Norfloxacin	Prokaryotic topo II inhibitor	*T. cruzi* and *B. culicis*	974	Thicker kDNA fibre strands in *B. culicis*
Enoxacin	Prokaryotic topo II inhibitor	*T. cruzi* and *B. culicis*	163.64	Thicker kDNA fibre strands in *B. culicis*
Berenil	DNA-binding drug	*T. cruzi* and *B. culicis*	20.25	Thicker kDNA fibre strands in *T. cruzi* and *B. culicis*
Distamycin	DNA-binding drug	*T. cruzi* and *B. culicis*	98.58	Thicker kDNA fibre strands in *B. culicis*

IC_{50}, 50% inhibitory concentration; topo, topoisomerase; kDNA, kinetoplast DNA.

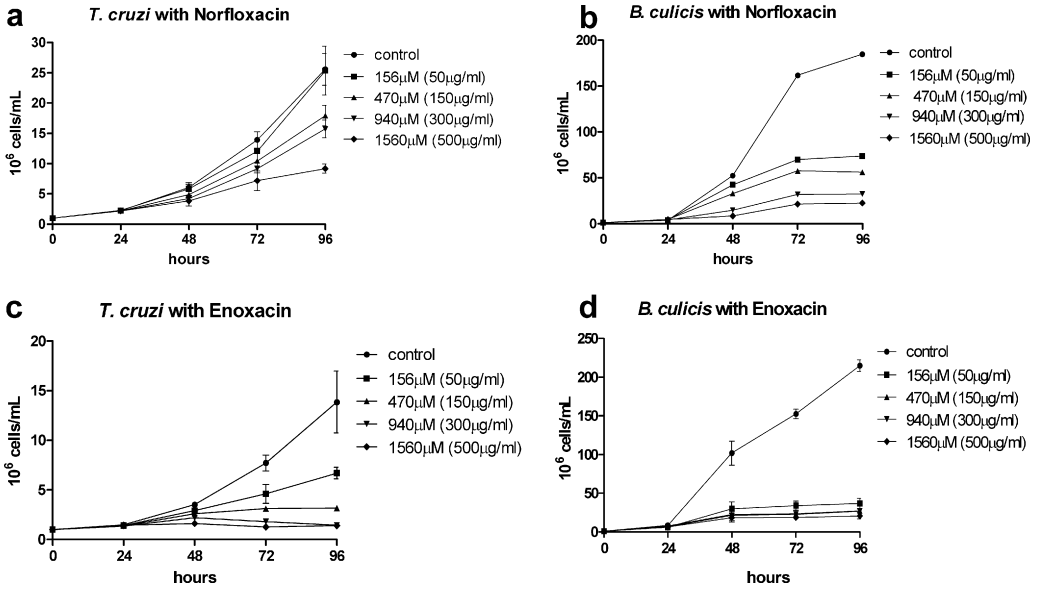

Fig. 5. (a and c) *Trypanosoma cruzi* and (b and d) *Blastocrithidia culicis* cell growth following treatment with the prokaryote topoisomerase II inhibitors norfloxacin (a and b) and enoxacin (c and d). Data are the average of three independent experiments.

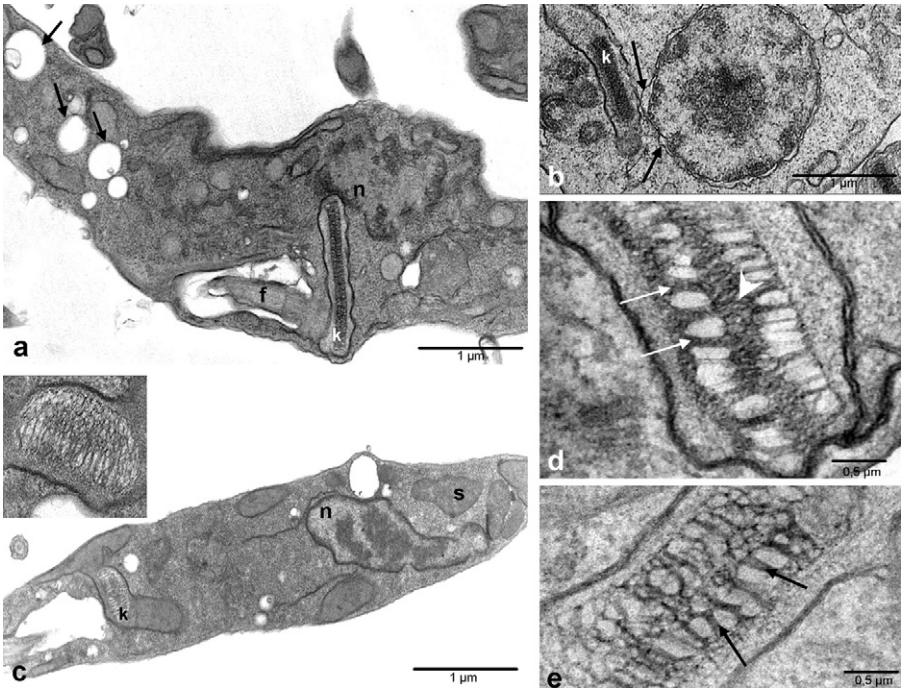

Fig. 6. (a) *Trypanosoma cruzi* treated with 470 μM norfloxacin for 48 h showed intense cytoplasmic vacuolisation (arrows). f, flagellum, n, nucleus, k, kinetoplast. (b) Treatment of *T. cruzi* with 156 μM enoxacin for 48 h promoted an enlarged space between the inner and outer membranes that enclose the nucleus (arrows). In both cases, *T. cruzi* did not show ultrastructural modifications either in kinetoplast DNA (kDNA) or nuclear DNA. (c) Ultrastructural aspects of non-treated *Blastocrithidia culicis* showing the kinetoplast (k), nucleus (n) and symbiont (s). The inset shows the looser kDNA network in this species. (d) *B. culicis* kinetoplast following treatment with 940 μM norfloxacin for 72 h. Note the formation of thicker DNA fibres (arrows) and also an electron-dense layer at the centre of the kinetoplast network (arrowhead). (e) Kinetoplast of *B. culicis* treated with 940 μM enoxacin for 48 h also presented thicker DNA fibres (arrows). However, lower kDNA compaction is observed compared with norfloxacin treatment.

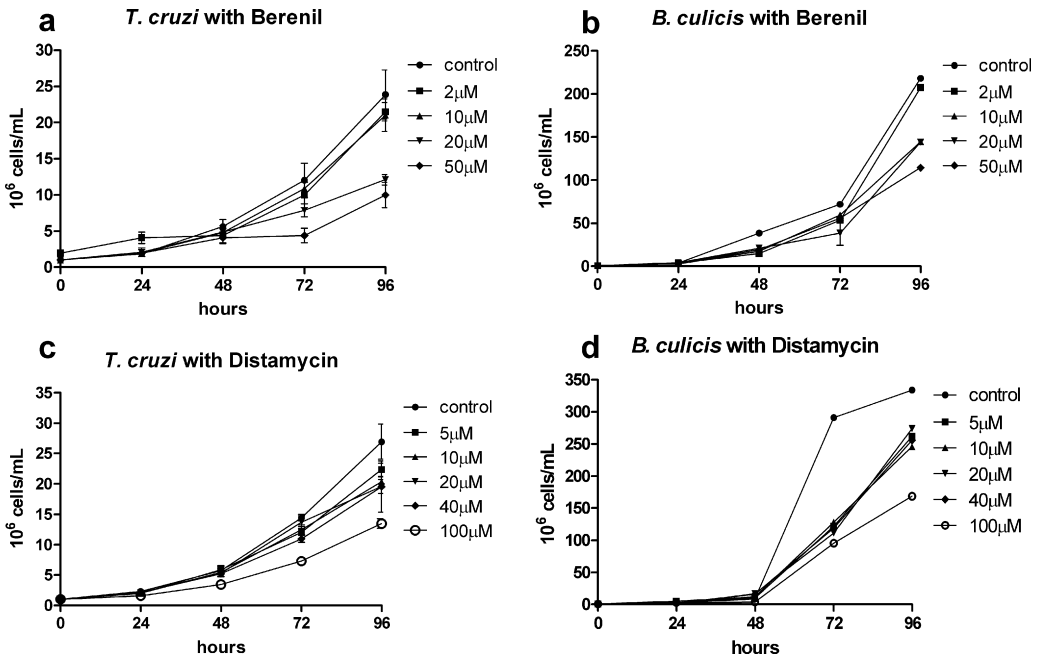

Fig. 7. (a and c) *Trypanosoma cruzi* and (b and d) *Blastocrithidia culicis* growth inhibition following treatment with the DNA-binding drugs berenil (a and b) and distamycin (c and d). Data are the average of three independent experiments.

proliferation. Rebeccamycin, which was tested for the first time in *T. cruzi*, was also effective in inhibiting protozoan growth, but had a higher IC_{50} compared with camptothecin.

A previous study reported successful treatment of the bloodstream form of *T. brucei* and *Leishmania* spp. with rebeccamycin, showing that low drug concentrations were able to reduce cell proliferation by up to 95% [30,53]. In this work, treatment with topo I inhibitors promoted severe unpacking of the heterochromatin in interphase cells, which is usually observed surrounding the nucleolus and juxtaposed to the inner nuclear membrane [7,54]. Such ultrastructural alterations were also observed in *T. cruzi* epimastigotes exposed to γ radiation [55]. Taken together, the effects of topo I inhibitors on growth impairment and nuclear structure alterations suggest that the *T. cruzi* cell cycle was arrested in order to repair the DNA. In this work, the kDNA ultrastructure was not affected by these inhibitors. Nonetheless, effects of camptothecin on mitochondrial DNA, such as minicircle linearization, have been reported in trypanosomatids [56].

Merbarone and mitoxantrone, eukaryotic topo II inhibitors, also promoted growth inhibition of *T. cruzi*. Mitoxantrone was one of the most effective compounds against protozoa proliferation, promoting cell growth impairment with a low IC_{50} value. Mitoxantrone and other eukaryote topo II inhibitors, such as etoposide and doxorubicin, have high trypanocidal activities on bloodstream forms of *T. brucei* [57]. Curiously, mitoxantrone did not affect the ultrastructure of the DNA-containing nucleus and kinetoplast. Previous results also showed that the eukaryotic topo II inhibitor novobiocin did not promote ultrastructural alteration either in the nucleus or in the kinetoplast of *T. cruzi* and *Leishmania amazonensis* [39]. Only reservosome content was reduced following treatment with mitoxantrone, which could be related to activation of the differentiation process of the epimastigote to the trypomastigote form, because cells are under stress when grown in the presence of this inhibitor. Since topoisomerase inhibitors have been described in other biological systems, it is important to highlight that in trypanosomatids these compounds may bind to other targets besides the expected one. Thus, some ultrastructural effects observed following drug treatment may not be related to enzyme inhibition.

Topo II activity is essential for nuclear and kinetoplast DNA replication and segregation. During mitosis, this enzyme accumulates at active centromeres, thus maintaining the kinetochore/centromere structure and promoting decatenation of sister chromatids, which involves double-stranded DNA cleavage [3]. Previous studies demonstrated that prokaryotic topo II inhibitors such as quinolones are able to block cell proliferation and promote ultrastructural alteration in trypanosomatid kinetoplasts [39,58]. In this study, norfloxacin and enoxacin, second-generation fluoroquinolones, were used, which specifically target bacterial topo II (gyrases) and topo IV by inducing cleavable complex formation, DNA fragmentation and cell death [59]. Enoxacin was more effective in blocking cell proliferation compared with norfloxacin, with an IC_{50} value that was six-fold lower in *T. cruzi*. In *Leishmania panamensis* promastigotes, nuclear topo II was 235-fold more sensitive to enoxacin than norfloxacin. *Blastocrithidia culicis* cell proliferation was more sensitive to prokaryotic topo II inhibitors than *T. cruzi*, similar to previous data on novobiocin and nalidixic acid treatment [39]. In *B. culicis*, both compounds induced compaction of the kDNA fibres, thus reinforcing the idea that the looser arrangement of the kDNA network makes this protozoan more susceptible to prokaryotic topo II inhibitors [39]. It is worth noting that the in vitro antitrypanosomal activity of fluoroquinolones varies widely according to species and stage development, presenting limited efficacy against *T. cruzi* and *L. donovani* amastigotes [60] and high cytotoxicity against bloodstream forms of *T. brucei* [61,62].

In this study, DNA-binding drugs did not have a significant effect on cell proliferation. However, berenil promoted striking alterations in the kinetoplast ultrastructure. In *T. cruzi*, prolonged treatment with high drug concentrations markedly affected the kDNA, which was invaded by invaginations of the inner mitochondrial membrane. Electron-lucid areas were observed around the kDNA disk and they may correspond to detached or linearized minicircles. This is in line with the proposal that berenil induces double-stranded cleavage of minicircles, presumably mediated by topo II inhibition [33]. Conversely, such electron-lucid areas may represent accumulation of RNA or proteins but not free circles, because berenil may be capable of inhibiting the release of mini-

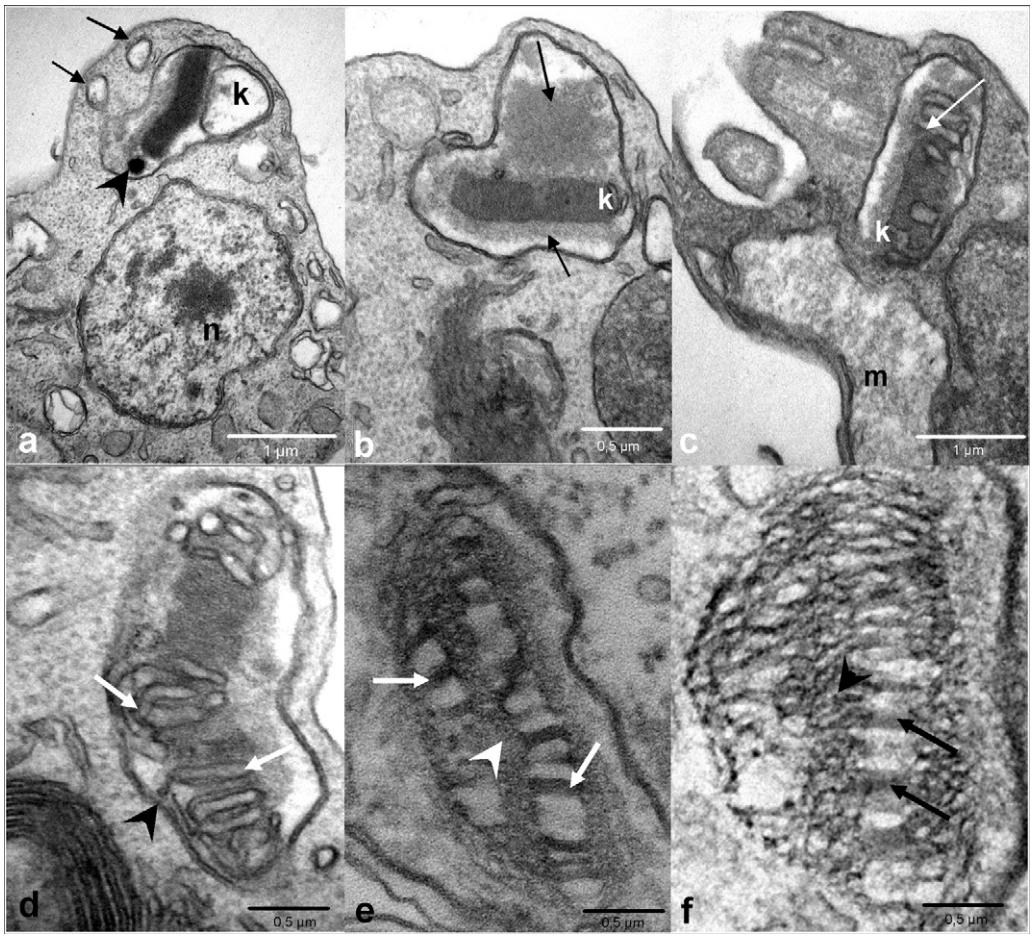

Fig. 8. (a) *Trypanosoma cruzi* treated with 10 μM berenil for 72 h presented mitochondrial swelling (arrows). An electron-dense point was observed close to the kinetoplast (k) antipodal sites (arrowhead). n, nucleus. (b) *T. cruzi* after treatment with 20 μM berenil for 48 h showed electron-lucid areas (arrows) near the kinetoplast disk. (c) Treatment of *T. cruzi* with 10 μM berenil for 72 h induced mitochondrion membrane profiles in the middle of the kinetoplast DNA (kDNA) (arrow), as well as loss of the mitochondrion matrix (m). (d) *T. cruzi* treated with 20 μM berenil for 48 h. With this higher magnification it is possible to note the membrane profiles (arrows), which in fact correspond to invaginations of the inner mitochondrial membrane (arrowhead). (e) *Blastocrithidia culicis* kinetoplast following treatment with 10 μM berenil for 72 h showed thicker DNA fibre strands (arrows and arrowhead) and a higher kDNA compaction compared with control cells. (f) Treatment of *B. culicis* with 100 μM distamycin for 72 h also promoted condensation of kDNA fibres (arrows and arrowhead).

circles for replication and topo II is unable to interact with DNA sites occupied by minor groove ligands [34,63]. In the present work, kinetoplast ultrastructural alteration in the *T. cruzi* epimastigote form treated with distamycin was not observed. However, intense kDNA network disruption was reported in the trypomastigote form of this protozoan after treatment with several aromatic diamidines [64]. *Blastocrithidia culicis* exhibited more condensed kDNA fibres following berenil treatment, and the effects were similar to those described with bacterial topo II inhibitors [39]. The distinct effects of berenil observed for each species may be related to the different kDNA arrangements that they contain [6,42].

The challenge in chemotherapeutic treatment is to target specific molecules in order to minimize host toxicity. In this context, topoisomerases are excellent candidates owing to their essential roles, especially in DNA replication, transcription and segregation. In this study, the major classes of topoisomerase inhibitors caused different physiological effects that can be attributed to factors such as permeability to cell membranes, resistance due to alterations in drug-binding sites of distinct enzymes, and pharmacokinetic properties. Further insight with the mechanism of action of topoisomerse inhibitors may pave way for development of selective drugs against pathogenic trypanosomatids.

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