Indotecan (LMP400) and AM13-55: Two novel indenoisoquinolines show potential for treating visceral leishmaniasis

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Abbreviations: NTD: Neglected Tropical Diseases; Top: DNA-topoisomerases; LiTopIB: Type IB Leishmania infantum Top; hTopIB: Type IB human Top; DALY: Disability-Adjusted Life Year; CPT: camptothecin; IFP1.4: Infrared Fluorescent Protein 1.4; FCS: foetal calf serum
ABSTRACT

Visceral leishmaniasis is an emerging neglected tropical disease (NTD) caused by the protozoan *Leishmania infantum* in the countries bordering the Mediterranean Basin. Currently there is no effective vaccine against this disease, and the therapeutic approach is based in toxic derivatives of SbV. Therefore, the discovery of new therapeutic targets and the development of drugs designed to inhibit them is an extremely important approach to fight this disease. DNA topoisomerases (Top) have been identified as promising targets against leishmaniasis. These enzymes are involved in solving topological problems generated during replication, transcription and recombination of DNA. Unlike the mammalian host, type IB DNA topoisomerase (TopIB) from *Leishmania* spp. is a unique bisubunit protein that makes it very interesting as a selective drug target. In the present investigation, we studied the effect of two TopIB poisons with indenoisoquinoline structure; Indotecan and AM13-55, on a murine BALB/c model of infected splenocytes with *L. infantum*, comparing their effectiveness with the clinically tested leishmanicide drug, paromomycin. Both compounds have high selectivity indexes when compared with uninfected splenocytes. SDS/KCl-precipitable DNA-protein complexes in *Leishmania* promastigotes and *in vitro* cleaving assays confirmed that these drugs are Top poisons. The inhibitory potency of both indenoisoquinolines on *L. infantum* recombinant TopIB was assessed *in vitro* showing that indotecan was the most active compound preventing the relaxation of supercoiled DNA. Experimental infections in susceptible BALB/c mice treated with 2.5 mg/kg body weight/day once every other day for a total of fifteen days showed that indotecan cleared more than 80% parasite burden of spleen and liver indicating promising activity against visceral leishmaniasis.
INTRODUCTION

Neglected Tropical Diseases (NTD) produced by vector-borne protozoa are mostly prevalent in underdeveloped and poor countries causing an estimated 4.1 million disability-adjusted life years (DALYs) lost (16). However, in developed countries where these diseases were unknown or had been eradicated for a long time, an unexpected new scenario has appeared. Increased tourist exchange, soldiers deployed in countries where these diseases are endemic, migratory fluxes and eventually climate changes, along with other pandemics, are challenging the apparent safety of rich populations (26,30). Since NTDs are prevalent in low-income countries, large pharmaceutical companies have neglected R & D of new drugs. Therefore, old compounds that are losing efficacy are still in use. These compounds have undesirable toxic effects and their dosage schedules are complex and repetitive, which compromises patient compliance (7).

Visceral leishmaniasis is a NTD increasingly affecting European countries by the massive flows of immigrants from North Africa (18). Its etiologic agent Leishmania infantum infects domestic dogs (canine leishmaniasis) that act as reservoir, and it is transmitted to humans through sandfly bites (20). For decades, the first-line treatment of this disease was the old-fashioned and toxic drugs derived from pentavalent antimony (SbV) (1). Recently, safer and more effective drugs such as amphotericin B, miltefosine and paromomycin are substituting SbV, despite the fact that they are not devoid of undesirable side effects and cannot be administered during pregnancy (8,9). Based on these reasons, the search for new compounds against this disease is very much needed.

Since the beginning of this century, DNA topoisomerase IB (TopIB) has been identified as a potential target against Leishmania and other trypanosomatids (3,5,6). The choice of this target is based on two main reasons: i) the enzyme has an increased expression during the rapidly dividing cycle of the pathogen, in a similar way to tumour cells, and especially ii) the pathogen’s enzyme is structurally different from that of the host (35).

Our research group found that L. donovani TopIB (LdTopIB) was atypically composed of two different subunits – each one encoded by different genes – that have to be assembled in the pathogen to reconstitute the active enzyme. One of the subunits contains the four amino acids of the active site, which is fully conserved from a phylogenetic point of view (35). The other subunit contains the catalytic amino acid (Tyr222), which acts by breaking one DNA strand by a specific nucleotide sequence.
All these features have also been described in the other protozoan-borne NTDs pathogens: Trypanosoma brucei – the agent responsible for sleeping sickness in Africa (11) – and T. cruzi – responsible for Chagas disease in South America (36). However, despite the fact that these enzymes conserve their catalytic domains unchanged, they display two non-conserved regions; one at the C-terminal end of the large protomer and the other at the N-terminal end of the small protomer, which are extremely important in sensitivity to topoisomerase poisons (12). These compounds act by stabilizing enzyme-DNA complexes, preventing the religation step and eventually producing single-strand breaks when they collide with the replication fork during DNA synthesis (22). The most studied TopIB poison is camptothecin (CPT) and its derivatives, including topotecan and the prodrug irinotecan, which are being used against certain tumours (32). Other non-CPT TopIB poisons are indolocarbazoles, such as the DNA intercalating drug rebeccamycin, and indenoisoquinolines, which were initially developed as antitumor compounds with an improved ability to stabilize cleavage complexes (2,25).

There are several promising indenoisoquinolines studied against tumour processes and more recently against experimental African trypanosomiasis (4). In this paper we show for the first time the effect of two indenoisoquinolines, indotecan and AM13-55, on the viability of free-living promastigotes and amastigote-infected murine splenocytes. For this purpose, we used a modified strain of L. infantum transfected with the gene encoding the infrared protein IFP1.4 from the extremophilic bacterium Deinococcus radiodurans (31). This fluorescent pathogen strain retains its virulence unaffected and allows studies of high performance in both free-living promastigotes and in ex vivo spleen explants where parasite growth conditions resemble natural infection environment. The results provided in this work demonstrate the high selectivity indexes of both molecules in vitro and the promising therapeutic potential of indotecan against visceral leishmaniasis.

MATERIALS AND METHODS

Reagents and culture media

Pyrococcus furiosus (Pfu), klenow polymerases and restriction enzymes were acquired from Roche (Roche Farma SA, Spain) and GE Healthcare (Spain). T4 DNA ligase was obtained from Stratagene (La Jolla, CA, USA). Cell culture media were purchased from...
Sigma-Aldrich (Spain). Indenoisoquinolines indotecan and AM13-55 (Fig. 1) were kindly provided by Dr. Mark Cushman, Dept. of Medicinal Chemistry and Molecular Pharmacology, Purdue University (USA). Primers for PCR amplification were from Sigma Genosys (UK).

**Generation of an infrared fluorescent *L. infantum* strain**

*L. infantum* promastigotes (BCN150 strain) were obtained by Dr. J. M. Requena (Centro de Biología Molecular “Severo Ochoa”, Madrid Spain). The 987 bp IFP1.4 coding region – an improved monomeric infrared fluorescent protein derived from *Deinococcus radiodurans* (31) – was amplified by PCR from IFP1.4_pcDNA3 vector – a gift from Dr. Roger Y. Tsien (Department of Pharmacology; Department of Chemistry and Biochemistry, University of California, San Diego, USA) – using the forward primer 5’CCGCTCGAGCCATGGCCACCATGGCTCGGGACCCTCTGCC3’ and the reverse primer 5’ATAAGAATGCGGCCGCTATTATACAGCTCGTCATCC3’.

The amplified fragment was digested with *Bgl*II and *Not*I and assembled into the expression vector pLEXSY-2-HYG (Jena Bioscience GmbH; Germany), which was previously digested with the same enzymes. The resultant plasmid was named pLEXSY-IFP1.4. Parasites were electroporated with the large *Swa*I targeting fragment from pLEXSY-IFP1.4 and selected on semisolid media as previously described (27). Many clonal lines were obtained, and correct integration into the 18s rRNA locus was confirmed by PCR analysis. The transformant strain was routinely cultured at 26 °C in M199 medium supplemented with 25 mM HEPES pH 6.9, 10 mM glutamine, 7.6 mM hemin, 0.1 mM adenosine, 0.01 mM folic acid, 1x RPMI 1640 vitamin mix (Sigma), 10% (v/v) heat-inactivated foetal calf serum (FCS) and antibiotic cocktail (50 U/ml penicillin, 50 μg/ml streptomycin).

**Ex vivo splenic explant cultures**

Balb/c mice were inoculated intraperitoneally with 10⁸ *L. infantum* purified metacyclic promastigotes. Briefly, infective promastigotes were isolated from stationary-phase culture by negative selection with peanut agglutinin (29). Five weeks post-infection spleens were aseptically dissected, washed in cold-PBS and placed in Petri dishes. Small pieces were obtained by using a scalpel. In order to obtain a single cell
suspension, tissue was incubated with 5 ml of 2 mg/ml collagenase D (Roche) prepared
in buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) for 20 min at 37 ºC. Cell suspension containing traces of spleen mass was gently
passed through a 100 μm cell strainer to remove tissue fragments (21). Splenocytes
were washed twice with PBS by centrifugation (500 x g for 7 min at 4 ºC) and re-
suspended in RPMI medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1x RPMI vitamins, 10 mM Hepes, and 50 U/ml penicillin and 50 μg/ml streptomycin at 37 ºC and 5% CO₂. Cells were counted and diluted at different cell densities. Cells were
seeded until confluence and different concentrations of the studied indenoisoquinolines
were administered to the explants for 48 h. The viability of infecting amastigotes was
assessed by registering the fluorescence emission at 708 nm in an Odyssey (Li-Cor)
infrared imaging system. The cytotoxicity of the drugs was assessed on uninfected ex
vivo explants after 48 h incubation, using the Alamar Blue staining method according to
manufacturer’s recommendations.

Recombinant Leishmania infantum TopIB (LiTopIB)

Cloning of LiTopIB ORFs (encoding large and small subunits), expression and
purification of the enzyme were carried out as previously described for LdTopIB (35).
Briefly, a Saccharomyces cerevisiae EKY3 strain deficient in TopIB activity [MAT α
ura3-52 his3Δ200 leu2 Δ1 trp1 Δ63 top1 ΔTRP1], was transformed by the lithium
acetate method (15) with the bicistronic pESC-URA vector, which carries both LiTopIB
ORFs. Single colonies were incubated overnight in SC-uracil media with 2% dextrose
(w/v). Since dextrose traces may interfere with the expression of the protein, yeast
cultures were incubated in SC-uracil media supplemented with 2% raffinose (w/v) for
24 h prior to a 6-h induction with 2% galactose (w/v). Yeasts were harvested, washed
with cold 1 x TEEG buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 10%
glycerol) and resuspended in 15 ml of the same buffer supplemented with 0.2 M KCl
and a protease inhibitors cocktail (Roche Farma SA, Spain). The cells were subjected to
one freeze/thaw cycle at 80 ºC and lysed by vortexing in a glass bead-beater at 4 ºC.
Protein extracts were obtained by centrifugation at 15,000 x g for 45 min at 4 ºC. Yeast
extracts were sequentially precipitated with two increasing concentrations of
ammonium sulfate (35 and 75%, respectively). The second precipitate supernatant was
loaded onto a P-11 phosphocellulose column (Whatman International Ltd. England),
which was equilibrated following manufacturer’s indications. LiTopIB protein was eluted at 4 ºC with a discontinuous gradient of KCl (0.2, 0.4, 0.6, 0.8 and 1 M) in TEEG buffer supplemented with 0.1 mg/ml sodium bisulphite, 0.8 mg/ml sodium bisulfite and the protease inhibitors cocktail. Active fractions were loaded onto a phenyl-sepharose column (Sigma-Aldrich) with a discontinuous inverse gradient of ammonium sulfate (1, 0.8, 0.6, 0.4 and 0.2 M). Elution was performed with subsequent Centricon (Millipore®) concentration.

**Supercoiled plasmid DNA TopIB-mediated cleavage assay**

The sensitivity of LiTopIB to indotecan, AM13-55 and CPT (used as control drug) was assayed by DNA cleavage assays. pBluescript SK(-) phagemid DNA (pSK) was used as nickable substrate. At least 100 units of purified LiTopIB were incubated with 0.5 µg of pSK DNA in 10 mM Tris-HCl buffer pH 7.5, 5 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 15 µg/ml bovine serum albumin and 50 mM KCl. Different drug concentrations in 1% (v/v) DMSO (20 µl reaction volume) were also added. Following incubation for 4 min at 25 ºC, reactions were then stopped with up to 1% (w/v) SDS and incubated in the presence of 1 mg/ml proteinase K for one extra hour at 37 ºC. Subsequently samples were extracted with one volume of phenol-chloroform and loaded onto a 1% agarose gel containing ethidium bromide to a final concentration of 40 pg/µl (17). The gel was run for 16 h at 4 V/cm and the images of cleavage products were acquired with a G-BOX (Syngene UK).

**DNA relaxation assays**

TopIB activity was assayed by the relaxation of negatively supercoiled plasmid DNA. One unit of recombinant LiTopIB was incubated with the corresponding drug for 15 min at 4 ºC. Then, the reaction mixture containing 0.5 µg of supercoiled pSK DNA, 10 mM Tris-HCl buffer pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 15 µg/mL bovine serum albumin, 50 mM KCl, in a total volume of 20 µl was added. Reaction mixtures were incubated for 4 min at 25 ºC. Enzyme reactions were stopped by the addition of up to 1% SDS (w/v) (final concentration) and digested with 1 mg/ml proteinase K at 37 ºC during one extra hour to remove the protein that remained linked to DNA fragments. The extent of plasmid DNA relaxation was assessed in 1% agarose gels by...
electrophoresis in 0.1 M Tris borate EDTA buffer (pH 8.0) at 2 V/cm for 16 h. Gels were visualized with UV illumination after ethidium bromide (0.5 μg/ml) staining. A further electrophoresis was run in the presence of 0.1 μg/ml ethidium bromide, in order to separate nicked DNA from relaxed topoisomers (33).

**SDS-KCl precipitation assay**

For indenoisoquinoline-induced protein-DNA complex determination, *L. infantum* promastigotes, previously labeled for 24 h with 0.5 μCi/ml [2-14C] thymidine, were exposed to different concentrations of CPT and indenoisoquinolines for 30 min (3). Cells were pelleted and lysed by incubation at 60 °C for 10 min in the presence of 1.25% (w/v) SDS; 0.4 mg/ml salmon sperm DNA and 5 mM EDTA. After the addition of 65 mM KCl, the reaction mixture was incubated on ice for 60 min. The precipitate was harvested by filtering through glass fiber paper (GF/C; Brandel Inc. MD, USA), pre-wetted with wash buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 100 mM KCl), washed (three times with 4 ml of wash buffer), and air-dried. After spotting 50 μl of the same density of labeled cell suspensions onto filter paper and precipitating nucleic acids using 5% trichloroacetic acid (v/v), total incorporation of radioactivity into DNA was measured by a liquid scintillation counter (Beckman LS600A). All assays included vehicle drug as control. The formation of DNA fragments, as a percentage of total labeled DNA, was calculated as follows: [(dpm in SDS-KCl drug – dpm in SDS-KCl solvent)/(dpm total incorporation)] x 100.

**Thymidine incorporation assay**

*Leishmania infantum* promastigotes (10 ml; 5 × 10⁶ cells/ml) were incubated with [2-14C] thymidine (0.5 μCi/ml) in the presence of drug or solvent in thymidine-free complete M199 medium. Five hundred μl aliquots were removed after 2, 4, 8, 10 and 24 hours and loaded onto glass fiber paper (GF/C; Brandel Inc. MD, USA). 14C-labeled-DNA was precipitated using ice-cold 5% (w/v) trichloroacetic acid and sequentially washed with PBS, 90% (v/v) ethanol and 70% (v/v) ethanol. Radioactivity was quantified by liquid scintillation. CPT was used as positive control.
Animals

Female BALB/c mice (age 4–6 weeks) were purchased from Harlan Ibérica Laboratories (Spain). All animal procedures were approved by the University of Leon subcommittee on Research Animal Care.

Infective-stage metacyclic \( L. \ infantum \) promastigotes were isolated from stationary phase cultures (6-day old) by negative selection with peanut agglutinin (29). Mice were infected with \( 10^7 \) metacyclic parasites intravenously through the tail vein. Fourteen days later, once every two-days for fifteen days, mice were administered a solution of indotecan or AM13-55 indenoisoquinolines diluted in DMSO (equivalent to a dose of 2.5 mg/kg of body weight per injection) intraperitoneally for a total of eight doses. Five days after the last treatment, the mice were euthanized, organs were removed and parasite loads were determined by limiting dilution assay (34). Briefly, a suspension of 20 mg of either spleen or liver was prepared by grinding the tissue in 1 ml of Schneider’s medium (Gibco BRL) containing 20% (v/v) heat-inactivated FCS in the presence of streptomycin and penicillin. This suspension was further diluted to reach a final concentration of 1 mg/ml. The parasite burden was determined by the limit dilution method after a 10 day period of parasite growth. Parasite burden was expressed as the number of free-living promastigotes per gram of wet tissue and compared to the non-treated group.

RESULTS AND DISCUSSION

Recent studies showed that the CPT derivatives topotecan, SN38, and especially gimatecan, are powerful leishmanicide agents. \textit{In vitro} assays demonstrated that they act by trapping both DNA and LiTopIB in reversible ternary complexes, producing precipitable SDS/KCl material when free-living promastigotes were incubated in the presence of these drugs (Prada et al., in press) (23). These compounds had good selectivity indexes when their cytotoxicity on mammalian cells (un-infected splenocytes) was compared to that from isolated \textit{ex vivo} splenic explants of BALB/c mice infected with \( L. \ infantum \). However, none of these compounds were more selective than miltefosine, the only effective oral treatment for visceral and cutaneous leishmaniasis (9). The use of a model of mouse splenocytes infected with a \( L. \ infantum \) strain expressing IFP1.4 permits high-throughput screening of compounds under
conditions that resemble those found in the animal, including the complete range of immune host cells, infected macrophages and fibroblasts (28).

**Screening of indenoisoquinolinic compounds**

The initial screening of new indenoisoquinolinic compounds was performed on free-living promastigotes; IRF1.4- *L. infantum* (BCN150) strain. To quantify cytotoxicity of the test compounds we utilized peritoneal BALB/c mouse macrophages. We first excluded compounds, that fell below an arbitrary cytotoxicity threshold in the peritoneal macrophage cultures (IC$_{50}$< 10 μM) (data not shown), and after exclusion of nine indenoisoquinolinic compounds (Fig. S1), only two new drugs were kept for further validation studies (Fig 1).

For these two compounds, anti-leishmanial activity (EC$_{50}$) was determined in the *ex vivo* splenic explant system. Comparison of these EC$_{50}$ values with cytotoxicity (IC$_{50}$) values in the un-infected *ex vivo* splenic explant allowed determination of an *in vitro* selectivity index (SI: IC$_{50}$/EC$_{50}$) (Table 1). As positive control we have used both paromomycin and CPT. Paromomycin has demonstrated a good selectivity index in the same system (Prada *et al.*, in press (23)).

CPT and both indenoisoquinolines (indotecan and AM13-55) were much more effective in preventing the proliferation of promastigotes and development of infection in splenocytes than paromomycin, an aminoglycoside antibiotic used in clinical practice against human leishmaniasis. The IC$_{50}$ of paromomycin was estimated to be 9.20 μM for mouse *ex vivo* splenic explants infected with *L. infantum*, whereas the IC$_{50}$ value was 0.1 μM for indotecan and AM13-55. Only CPT was more effective (IC$_{50}$ = 0.03 μM) at killing the parasites. To estimate the cytotoxicity on mammalian cells and therefore, to determine selectivity indexes of the studied compounds, we prepared non-infected *ex vivo* splenic explant. These cells were exposed to different concentration ranges of the drugs for 48 h and the viability was determined by the Alamar Blue method. Dose response curves were fitted by nonlinear analysis with the Sigma-Plot statistical package, showing that the least toxic compound was paromomycin (IC$_{50}$ = 15.70 mM), followed by indotecan (IC$_{50}$ = 57.16 μM) and AM13-55 (IC$_{50}$ = 48.37 μM). Selectivity indexes of each compound were calculated as the ratio between the IC$_{50}$ values on the uninfected explants system vs. the IC$_{50}$ values on infected *ex vivo* splenic explants. Both
Indenoisoquinolines have very high selectivity indexes; 571.6 for indotecan and 483.7 for AM13-55, which are very much higher than CPT-related compounds and miltefosine (23), but less than paromomycin (the safest compound tested with a selectivity index of 1706.5).

**Indotecan and AM13-55 induce TopI-DNA covalent complexes**

Figure 2A shows the induction of DNA cleavage complexes by indotecan and AM13-55 in the presence of TopI as tested in supercoiled plasmid DNA (pSK). Both indotecan and AM13-55 induce DNA cleavage complexes in a similar pattern to CPT, but with differences in their relative intensities. The indotecan cleavage complex showed the same strong intensity than CPT at 100 μM, although the cleavage complex intensity is almost the same in the range of 0.1 to 10 μM. On the other hand, the AM13-55 cleavage complex has lower intensities as CPT but the pattern resembles CPT, being proportional with drug concentration. We observed a dose-dependent increase in nicked DNA for the three compounds, thus confirming the TopIB poisoning nature of both indenoisoquinolines (2,4).

We also studied the potency to inhibit the DNA relaxation activity, comparing the human and leishmania TopIB enzymes. The LiTopIB enzyme was more sensitive to indotecan and AM13-55 than the human enzyme. Figure 2B (top panel) showed that Leishmania TopIB was already inhibited at 80 nM indotecan. However, 2.5 μM were required to achieve the same effect on the human enzyme. AM13-55 (Fig 2B lower panel) was a little bit less efficient, inhibiting the *Leishmania* TopIB at 0.15 μM, while the human enzyme was not inhibited until 2.5 μM.

These results suggest that indenoisoquinolines indotocan and AM13-55 at pharmacologically relevant doses are primarily TopI poisons with DNA cleavage patterns exhibiting similarities and differences from those of CPT, but they are much more effective in *Leishmania* than in human enzyme.
Induction of TopI-DNA complexes by indotecan and AM13-55 in *L. infantum* cultures

To determine whether indotecan and AM13-55 induce TopI cleavage complexes in drug-treated cells, we used the SDS-KCl precipitation assay. IFP1.4-*L. infantum* (BCN150) promastigotes were exposed for 30 min to different concentrations of CPT (used as positive control), indotecan and AM13-55 in a concentration range from 0.1 to 90 μM. After this time, stabilized protein-DNA complexes were quantified by the SDS/KCl precipitation method. Fig. 3A shows that CPT produced an increasing amount of SDS/KCl-precipitable complexes that were dependent on the concentration of drug that was added up to a value close to 80% of total labeled DNA. None of the indenoisoquinolines studied had such potent effect within the same concentration range. Indotecan and AM13-55 clearly showed an increase in the amount of SDS/KCl-precipitable complexes, but unlike CPT, indotecan produced up to 20% labeled DNA at 10 μM. AM13-55 reached up to 40% at the same concentration. These results are higher than those obtained by Bakshi and co-workers with three sets of indenoisoquinolines against trypomastigotes of *T. brucei* (4). In that case, the indenoisoquinolines tested only induced up to 12% of cleavage complexes from the total labeled DNA.

Since indotecan and AM13-55 inhibit DNA relaxation and induce stabilization of DNA cleavage complexes, we performed a competition study between CPT and both indenoisoquinolines in order to evaluate the primary mediator of cell killing in Leishmania. Fig. 3B shows that 5 μM of both tested compounds were not able to prevent CPT-mediated TopIβ-DNA stabilization thus pointing to a poison nature of both drugs rather than interaction to DNA.

In order to assess the arresting effect of indotecan and AM13-55 on DNA synthesis, 5 × 10⁶ exponentially growing *L. infantum* promastigotes were pulsed with 0.5 μCi [2-¹⁴C] thymidine in the presence of 1 μM CPT, 1 μM AM13-55, 1 μM indotecan or solvent (control). After a time lapse of 2, 4, 6, 8, 10 and 24 h, labeled DNA was determined by scintillation counting. All the TopI poisons arrested DNA synthesis at all time points in more than 90% with no significant differences amongst them (Fig. 3C). This results correlate well with those found by Cushman *et al.* in 2011. They showed that indenoisoquiones with large primary amines side chain like indotecan and AM13-55 do not intercalate into free DNA and do not suppress cleavage complex formation at high concentration, unlike those with small primary amine (4,19).
Indotecan and AM13-55 used as therapeutic agents in a visceral leishmaniasis murine model infected with *L. infantum*

To evaluate the therapeutic potential of indotecan and AM13-55 *in vivo*, we performed an experimental infection in a susceptible BALB/c strain mouse with *L. infantum* promastigotes (wild type strain). Seven million infective-stage metacyclic promastigotes were administered intravenously by the caudal vein of 15 healthy mice that were four to six weeks old. By day 15 post-parasite inoculation, mice were divided into three groups of five animals each. Mice were treated with 0.5 ml solutions of indotecan or AM13-55 in DMSO/saline, equivalent to 2.5 mg/kg body weight per injection, intraperitoneally every two days for 15 days (total eight doses). As control, indenoisoquinoline vehicle was administered.

Five days after the last administration, animals were killed by cervical dislocation and spleens and livers were aseptically dissected to determine the parasite load. This was determined by the limiting dilution assay. Each treatment group was compared to the control, which had received only the vehicle in which the drug had been dissolved.

Fig. 4A shows the parasite burden in the group of mice treated with 2.5 mg/kg of indotecan in spleen (upper panel) and liver (lower panel). After the administration schedule was completed, a drastic reduction (*p*<0.001) of the number of transforming amastigotes recovered from the target organs of drug-treated animals was observed. On the other hand, mice treated with the same dose of AM13-55 (fig 4B) showed that only spleen was efficiently cleared of infecting parasites, unlike liver, which retained the pathogen load at similar levels to the untreated group (Fig 4B). In a subsequent trial, 5.0 mg/kg of body weight of AM13-55 was administered under the same conditions as previous experiments (data not shown). Parasite load was reduced above 90% in the spleen, but not in the liver, which remained unchanged. The resistance of liver macrophages to kill the parasites may be due to metabolic transformations of the parent compound in the hepatic parenchyma on TopIB-inactive byproducts.

Despite the fact that no definitive cure was achieved with indotecan, our results are very similar to those found with 20 mg/kg body weight of paromomycin alone or in combination with 200 mg/kg body weight glucantime on experimental infections of BALB/c visceral leishmaniasis (14). Furthermore, both indotecan and AM13-55 were...
more effective than CPT (2.5 mg/kg body weight), both free and liposome-encapsulated, in a murine model of *L. donovani* leishmaniasis. The authors of that study found that parasite load in livers and spleens were just reduced a 55% average when infected mice were treated with this TopI poison (24).

In conclusion, we have proven that the two indenoisoquinolines analyzed have strong leishmanicidal activity both *in vitro* (*ex vivo* splenic explant cultures) and *in vivo* (visceral leishmaniosis murine model). These compounds act by stabilizing the DNA-LiTopIB cleavage complexes and inhibiting the intrinsic relaxation activity of human and leishmanial TopIB enzymes. Indotecan has a very high selectivity index with respect to host cells and depleted the parasitic burden of spleen and liver. These facts make this compound an exceptional candidate for its development as new leishmanicidal drug with a better therapeutic profile than others currently in use.

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CAPTIONS TO FIGURES

Figure 1. Chemical structures of the indenoisoquinolines indotecan and AM13-55 used in the present study.

Figure 2. Indotecan and AM13-55 induce TopI-DNA covalent complexes and inhibit supercoiled DNA relaxation. A) Supercoiled plasmid DNA TopIB-mediated cleavage assay showing the displacement towards cleavage complexes mediated by both indenoisoquinolines and CPT on LiTopIB. Samples were run on a 1% agarose gel containing ethidium bromide to a final concentration of 40 pg/µl in order to separate supercoiled and relaxed DNA. The results are representative of three independent trials. B) Inhibition of supercoiled DNA relaxation by indotecan (top gel) and AM13-55 (bottom gel) mediated by human (left lanes) and leishmanial TopIB (right lanes). Reaction mixtures were incubated at 37 °C in a final concentration of 150 μM KCl and then stopped with SDS up to a final concentration of 1% of reaction volume. Products were resolved in a 1% agarose gel and visualized by ethidium bromide staining. The results are representative of three independent trials.

Figure 3. Induction of TopI-DNA complexes by indotecan and AM13-55 in L. infantum cultures and DNA synthesis inhibition. SDS/KCl-precipitable enzyme-DNA complexes at increasing concentrations of the drugs under study: A) CPT, indotecan and AM13-55, in promastigotes of L. infantum after 30 min growth in the presence of different concentrations of drugs. Notice that in CPT the x-scale is up to 10 μM, whereas in panels indotecan and AM13-55 is up to 90 μM. Results are expressed as mean ± SE of at least three different experiments in duplicate; B) indotecan and AM13-55 increase CPT-induced cleavable complex formation. Leishmania promastigotes were treated with 5 μM indotecan, 5 μM AM13-55 or 10 μM CPT for 30 min, or with 5 μM indotecan or AM13-55 for 5 min prior to addition of 10 μM CPT, followed by an additional 25-min incubation; (*) P<0.01; (**) P<0.05 using paired t-Student test; C) [2-14C] thymidine incorporation into DNA of growing promastigotes in the presence of 1 μM CPT (empty dots), 1 μM AM13-55 (empty triangles), 1 μM indotecan (solid triangles) or solvent (solid dots). The amount of labeled DNA is expressed as total cpm per assay at different time points (2, 4, 6, 8, 10 and 24 h). Results are expressed as mean ± SE of at least three different experiments in duplicate.
Figure 4. Indenoisoquinolines cleared tissue parasitic burden in experimentally infected BALB/c mice with *L. infantum*. Three groups of five animals each were challenged with $10^7$ metacyclic promastigotes administered by the caudal vein. Fifteen days after infection animals were injected intraperitoneally every two days for a total of fifteen days (eight doses total), with a dose of 2.5-mg/kg body weight per injection of A) indotecan, B) AM13-55 or the corresponding vehicle (a solution of DMSO in sterilized saline solution). Mice were killed five days after the last treatment and the spleens and livers were aseptically removed, weighted and homogenized in medium supplemented with 20% (v/v) FBS. After 10 days, transforming promastigotes were counted and the limit dilution was considered for determining the parasitic burden of each organ. The results are representative of two independent trials; statistical differences were observed between groups (*) $P<0.001$ using paired t-Student test.
REFERENCES


Table 1. IC₅₀ calculation after a 48-h period of exposure to the selected compounds of *L. infantum* promastigotes, *ex vivo* infected splenocytes and uninfected splenocyte under methods described in the corresponding section. IC₅₀ at 48 h of the compounds selected in freshly uninfected splenocyte culture and values of SI₄₈h between this cell line and infected splenocytes with *L. infantum* amastigotes *ex vivo* were calculated from the dose-response curves performed in triplicate in separate experiments after performing a nonlinear fitting with the SigmaPlot® program.

<table>
<thead>
<tr>
<th>drug</th>
<th>IC₅₀ <em>L. infantum</em> promastigotes</th>
<th>IC₅₀ amastigotes</th>
<th>IC₅₀ uninfected splenocyte culture</th>
<th>SI₄₈h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paromomycin</td>
<td>42.41 µM ± 1.65</td>
<td>9.20 µM ± 0.01</td>
<td>15.70 mM ± 3.54</td>
<td>1706.5</td>
</tr>
<tr>
<td>Camptothecin (22)</td>
<td>1.12 µM ± 0.13</td>
<td>0.03 µM ± 0.01</td>
<td>0.62 µM ± 0.13</td>
<td>20.7</td>
</tr>
<tr>
<td>Indotecan</td>
<td>0.10 µM ± 0.08</td>
<td>0.10 µM ± 0.05</td>
<td>57.16 µM ± 6.01</td>
<td>571.6</td>
</tr>
<tr>
<td>AM13-55</td>
<td>1.02 µM ± 0.09</td>
<td>0.10 µM ± 0.37</td>
<td>48.37 µM ± 3.68</td>
<td>483.7</td>
</tr>
</tbody>
</table>

* selectivity index; SI₄₈h = IC₅₀ amastigotes / IC₅₀ infected splenocytes
A

\[
\text{AM13-55}
\]

B

\[
\text{indotecan (LMP400)}
\]