Consequence of Hoechst 33342-mediated *Leishmania* DNA topoisomerase-I inhibition on parasite replication

J.-F. MARQUIS[1], M. DROLET[2] *and* M. OLIVIER[1]*

[1] *Centre de Recherche en Infectiologie du CHUQ, Département de Biologie Médicale, Faculté de Médecine, Université Laval, Sainte-Foy, Québec, Canada, G1V 4G2*
[2] *Département de Microbiologie et Immunologie, Université de Montréal, CP 6128, Succursale Centre-Ville, Montréal, Québec, Canada, H3C 3J7*

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SUMMARY

This study reports that inhibition of *Leishmania* Topo-I with the minor groove-binding ligands (MGBLs) Hoechst 33342 (Ho342) blocks parasite growth in culture by mechanisms involving DNA breakage. While Ho342 inhibited the replication of several species of *Leishmania* in a dose- and time-dependent manner, Ho258 was not effective. Cytofluorometric analysis suggested that superior effectiveness of Ho342 over Ho258 was attributed to *Leishmania* parasites being more permeable toward Ho342. This observation was supported by the ability of both Ho342 and Ho258 to block the relaxation of supercoiled pBR322 DNA by *Leishmania* Topo-I. The Ho342 specificity toward *L. donovani* Topo-I was reinforced by the observation that increased Topo-I gene expression and Topo-I activity in *Leishmania* was paralleled by augmented resistance for this compound. Furthermore, the capacity of NaCl treatment to reverse MGBL-mediated DNA break suggests that Ho342 targetted Topo-I. Moreover, we observed that Ho342-inducible arrest of *Leishmania* growth was accompanied by G_1 arrest and induction of cell death that closely resembles apoptosis. Taken together, our results suggest that MGBL compounds show promise as Topo-I inhibitors against *Leishmania* infection.

Key words: *Leishmania*, topoisomerase-I, MGBLs, Hoechst 33342, DNA replication, cell death.

INTRODUCTION

The sandfly-transmitted, intracellular protozoan parasite *Leishmania* spp. infects several million people worldwide (Herwaldt, 1999). Individuals infected with this microorganism develop diverse pathologies encompassing cutaneous, muco-cutaneous, and fatal visceral afflictions. The lack of a vaccine, difficulties in vector control, and the development of parasite resistance toward drug therapies, all contribute to the rise in incidence of leishmaniasis (Herwaldt, 1999).

DNA topoisomerases (Wang, 1996) represent a unique class of enzymes that alter DNA topology by breaking and rejoining the DNA phosphodiester backbone (Liu, 1989; D'Arpa & Liu, 1989; Wang, 1985). Topoisomerase enzymes participate in several cellular metabolic processes associated with complementary DNA (cDNA) strand separation (i.e. replication, transcription, recombination and repair). Type I and type II topoisomerases (Topo-I and Topo-II respectively), are classified according to their mode of cleaving DNA (Liu, 1989). Thus, Topo-I catalyses DNA relaxation via a transient single-stranded DNA break, whereas Topo-II catalyses the topological crossing of double-stranded DNA segments by a transient double-stranded DNA break (Liu, 1989). Topo-I is an important enzyme in the regulation of DNA functions (Wang, 1985, 1991). It has been detected in many trypanosomatidae (Melendy & Ray, 1987), and it was purified from *Trypanosoma cruzi* (Riou *et al*. 1983) and *Leishmania donovani* (Chakraborty, Gupta & Majumder, 1993). Of interest, results obtained with the Topo-I inhibitor camptothecin, revealed that Topo-I activity was detectable in both the mitochondrion and the nucleus of these parasites (Bodley & Shapiro, 1995). More recently, we have cloned a Topo-I-like gene (1905 bases encoding a putative protein of 635 amino acid residues) from *L. donovani* (Broccoli *et al*. 1999). A substantial part of this protein shares significant homology with the highly conserved core domain of other known members of the IB (eukaryotic) topoisomerase family (Broccoli *et al*. 1999).

Several DNA minor groove-binding ligands (MGBLs) have been reported to exhibit anti-tumour activity and to act as Topo-I inhibitors (Chen *et al*. 1993*a*, *b*). Indeed, compounds such *bis*benzimidazoles Hoechst 33342 (Ho342) and Hoechst 33258 (Ho258), and various terbenzimidazole derivatives, whose primary mode of DNA binding is through an interaction with the DNA minor groove (Parkinson *et al*. 1990; Pilch *et al*. 1996; Pjura, Grzeskowiak & Dickerson, 1987; Spink *et al*. 1994), have been identified as specific Topo-I inhibitors (Chen *et al*.

* Corresponding author: Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, RC-709, 2705 boul. Laurier, Ste-Foy (Québec), Canada G1V 4G2. Tel: +418 654 2705. Fax: +418 654 2715. E-mail: martin.olivier@crchul.ulaval.ca

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1993*a*, *b*; Kim *et al*. 1996*b*; Sun *et al*. 1994, 1995). Moreover, the classical anti-trypanosomal drug berenil has been found to be a MGBL targetting Topo-I and to induce Topo-I-cleavable complex (Chen *et al*. 1993*b*). The MGBLs exert their principal action by interfering with the catalytic cycle of this enzyme during the creation of Topo-I-linked DNA breaks, thus leading to arrest of replication (Chen *et al*. 1993*b*). It is the stabilization of the cleavable complex that is responsible for DNA fragmentation and cytotoxicity (Liu, 1989).

Previous studies have reported that Ho342 and Ho258 were among the most effective MGBL compounds to induce Topo-I-cleavable complexes (Chen *et al*. 1993*b*; Kim *et al*. 1996*a*; Sun *et al*. 1994). Moreover, these Hoechst compounds have a fluorescent property that enables uptake of the drug to be followed directly in the parasites. In light of these observations, we became particularly interested in testing the toxicity of the Ho342 [2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride trihydrate] and Ho258 [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperizinyl)-2,5′-bi-1*H*-benzimidazole trihydrochloride pentahydrate]. We further attempted to corroborate this toxicity with their ability to create Topo-I-linked DNA breaks and ultimately lead to inhibition of parasite growth. Further, our findings may help to establish the mechanisms by which Topo-I inhibitors, such as MGBL compounds, affect the replication of protozoan pathogens.

MATERIALS AND METHODS

Drug solutions

Ho342 and Ho258 molecules were purchased from Sigma, and etoposide from Biomol. All stock solutions (10 mM) in dimethyl sulfoxide (ICN Biomedicals, Inc.) were stored at −20 °C.

Parasite cultures

L. donovani strain 1S2D (MHOM/SD/00/LS), *L. mexicana mexicana* (MNYC/BZ/62/M379), *L. major* Friedlin (MHOM/IL/80/FRIEDLIN), *L. braziliensis panamensis* (strain 1077) promastigotes, and *Trypanosoma brucei* (strain 427) trypomastigotes were grown at room temperature and transferred biweekly in SDM-79 culture medium (SDM) supplemented with 10% (v/v) foetal bovine serum (FBS) as previously described (Olivier & Tanner, 1987; White *et al*. 1988). *L. donovani* strain 1S2D was previously described (Lamontagne & Papadopoulou, 1999). *L. major* Friedlin and *T. brucei* were provided by Dr Marc Ouellette from Université Laval, Québec, Canada. *L. mexicana mexicana* and *L. braziliensis panamensis* were obtained from the ATCC. Resistant parasites (*L. donovani* strain 1S2D) were selected for Ho342 resistance by stepwise increase with 10, 20, 30, 40, and 50 μM Ho342.

Topo-I treatment

To monitor the impact of Topo-I inhibitors on *Leishmania* growth, parasites were transferred (10^7 log phase promastigotes) into 5 ml of SDM in the absence or presence of increasing concentrations of Ho342 and Ho258 (0 to 100 μM). Protozoan growth was monitored over 6 days by measuring the absorbance at 600 nm using an automated microplate reader (Organon Teknika, Reader 510) (White *et al*. 1988). The data presented are representative of at least 3 experiments carried out in duplicate.

Drug permeability

L. donovani log phase promastigotes (5×10^6 parasites/ml) treated or not with Ho342 and Ho258 (0–125 μM) were collected after 1 h of treatment and cell nuclei harvested, based on the method of nuclear extraction previously described (Andrews & Faller, 1991). Briefly, the parasites were pelleted in a microfuge (10 sec, max speed) and resuspended in 400 μl of cold Buffer A (10 mM HEPES-KOH, pH 7·9 at 4 °C, 1·5 mM $MgCl_2$, 10 mM KCl, 0·5 mM dithiothreitol, 0·2 mM PMSF) by flicking the tube. After this step, the parasites were allowed to swell on ice for 10 min, and then vortexed for 10 sec. Samples were then pelleted in a microfuge (10 sec, max speed), and the supernatant fraction discarded. Resulting pellets were resuspended in 500 μl of cold phosphate-buffered saline (PBS). The fluorescent property of the Hoechst compounds enabled the intensities of the fluorescence of the nuclear extract to be directly measured by flow cytometry with an Epics Elite ESP (Coulter Electronics, Miami, Florida). The samples were analysed by gating the system on nucleus size to avoid misinterpretation of the results with other cellular contaminants. The data presented are representative of a minimum of 3 experiments independently performed in quadruplicate.

Topo-I activity assays

L. donovani extracts were obtained from 5×10^8 log phase promastigotes, and submitted to the Topo-I activity assays (Tosh *et al*. 1999). Briefly, the parasites were resuspended in 100–200 μl of Topo-I assay buffer (10 mM Tris–HCl pH 7·9, 1 mM EDTA, 150 mM NaCl, 0·1% BSA, 0·1 mM spermidine, 5% glycerol), and lysed by repeated passage through a 25-gauge needle. The parasite extracts (2 μg of total proteins) were incubated for 1 h at 37 °C in a 20 μl volume containing Topo-I assay buffer and 0·5 μg of supercoiled pBR322 (Boehringer Mannheim). They were further incubated with 0·5 μg/μl proteinase K, 1% SDS, and 100 mM EDTA for 30 min at 50 °C

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before the addition of 2·5 μl of stop mix (5% sarkosyl, 0·0025% bromophenol blue, 25% glycerol). Supercoiled and relaxed forms of pBR322 were separated in a 1% agarose slab. After migration in 1 × TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA pH 8·0), the gel was soaked in ethidium bromide, and UV illuminated to reveal the status of DNA coiling. To exclude the possibility of Topo-II activity, Mg^{2+} ions and ATP were omitted from the reaction mix, both of which are necessary for Topo-II activity (Liu, Liu & Alberts, 1979; Stetler, King & Huang, 1979). All assays were repeated 3 times.

Topo-I gene expression

Total RNA from 2×10^6 *L. donovani* log phase promastigotes (wild-type and resistant) were extracted using TRIzol reagent (Gibco BRL). Briefly, RNA was resolved on a 1% agarose gel and transferred to a Nytran® Plus nylon membrane. After transfer of material, the membrane was UV-exposed for 3 min on a transilluminator, and pre-hybridized for 4 h at 42 °C in a solution of 5 × SSC, 10 × Denhardt's solution, 50 mM $NaPO_4$, 0·2 mM dextran sulfate, 0·5% SDS, 133 mM glycine, 50% formamide with 150 μg/ml of salmon sperm DNA. The membrane hybridization was performed overnight at 42 °C with a [α-^{32}P]dCTP-labelled *Sac*I DNA fragment from the *L. donovani TOP1*-like gene, as we previously described (Broccoli *et al*. 1999). After incubation, the membrane was washed 3 times with 2 × SSC/0·1% SDS and 3 times with 0·1 × SSC/0·1% SDS (15 min per wash, 42 °C). Autoradiography was performed using X-ray film (Kodak). The data presented represent 1 out of 3 monitored experiments performed independently.

DNA fragmentation analysis

Initially *L. donovani* promastigotes ($3–5 \times 10^7$/ml) were radio-isotope labelled overnight with [^{3}H]thymidine (specific activity, 50 Ci/mmol) at a final concentration of 50 μCi/ml. Labelled parasites were then washed 3 times with PBS, pelleted by centrifugation (2500 rpm, 5 min), and resuspended in culture medium at a final concentration of 10^7 cells/ml. The parasites were next incubated in the presence of increasing concentrations of Topo-I inhibitors (0–25 μM) for 30 min at room temperature. After incubation, the cells were pelleted at room temperature by centrifugation (2500 rpm, 5 min), and then lysed as previously described for the K^+-SDS procedure (Rowe *et al*. 1986). Resulting radioactive protein–DNA complexes from this step were measured with a scintillation counter (Beckman, LS 6000 TA). Reversion of the cleavable complexes was tested with 0·5 M NaCl on the parasites before lysis with SDS. The data obtained are representative of a minimum of 3 experiments performed in quadruplicate.

Cell cycle analysis

Hoechst-treated *L. donovani* (5×10^6 promastigotes/ml) were collected from log phase cultures at different time-points over 48 h and analysed for DNA content as previously described (Gale, Carter & Parsons, 1994), with some modifications. Briefly, the cells were fixed in 70% methanol/PBS (1 h, −20 °C). Following this, the cells were washed once in PBS, resuspended in PBS, and incubated for 20 min at 37 °C in the presence of 10 μg/ml RNase A. Propidium iodide (95–98%; Sigma) was added to the fixed cells at a final concentration of 100 μg/ml and incubated for 1 h at 37 °C. The cell populations ($< G_1$, G_0/G_1, S and G_2M) were analysed by flow cytometry using an Epics XL (Coulter Electronics, Miami, Florida) to estimate the DNA content of each cell. The data presented are representative of 1 out of 6 experiments independently performed.

Quantitative measurement of apoptosis

L. donovani (5×10^6 promastigotes/ml) were treated or not with Ho342 and Ho258 at a final concentration of 25 μM. The cells were collected at different time-points over 48 h and treated as described previously (Pollack & Ciancio, 1990). Briefly, the cells were pelleted by centrifugation (2500 rpm, 5 min), stained with 50 μl of propidium iodide (20 μg/ml) and kept on ice for 30 min. Cells were then fixed in 950 μl of 25% ethanol/PBS, and stained on ice for at least 30 min with 25 μl of Ho342 at 200 μM. The analysis of fluorescence intensity for each sample was evaluated with an Epics Elite ESP (Coulter Electronics, Miami, Florida). The data presented are representative of a minimum of 3 experiments independently performed.

Qualitative measurement of apoptosis

L. donovani (5×10^8 promastigotes) were treated or not with Ho342 (10, 25, and 50 μM) or hydrogen peroxide (0·5 mM and 1 mM H_2O_2; Sigma), and DNA extracted as previously described (Wang, 1985, 1991). Briefly, parasite pellets were treated with Triton lysis buffer (10 mM Tris, 10 mM EDTA, and 0·5% Triton X-100, pH 7·4) for 30 min at 4 °C. Proteinase K (20 μg/ml final) was added, and the resulting lysates incubated for 2 h at 50 °C. After centrifugation at 18 000 ***g*** for 30 min at 4 °C, DNA from supernatant fractions was purified by phenol–chloroform–isoamyl extraction method and precipitated with 0·5 M NaCl (final concentration) and 1 volume of isopropanol. The resulting DNA was washed with 70% ethanol, air dried, and solubilized in distilled water. RNase A (0·3 mg/ml) treatment was given for 30 min at 4 °C. Then, the amount of DNA was determined spectrophotometrically at 260/280 nm, mixed (10 μg of DNA/lane) with tracking dye, and loaded on 1%

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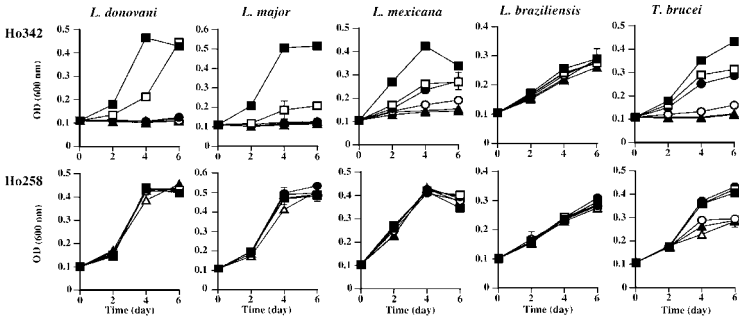

Fig. 1. Effects of Topo-I poisons on the growth of parasites. Parasites were grown in the presence of increasing concentrations of Ho342 or Ho258. Nil (■); 5 μM (□); 10 μM (●); 25 μM (○); 50 μM (▲); 100 μM (△). Optical density (OD) was followed for 6 days (mean ± S.E.M.).

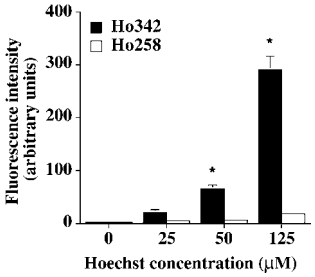

Fig. 2. Hoechst nuclear permeability. Parasites were treated with 25, 50, and 125 μM of Ho342 and Ho258 for 1 h. The nuclei were extracted as described in the Materials and Methods section, and the fluorescence intensity measured by flow cytometry. The results are expressed in arbitrary units (mean ± S.E.M.). *Significant differences ($P < 0{\cdot}05$).

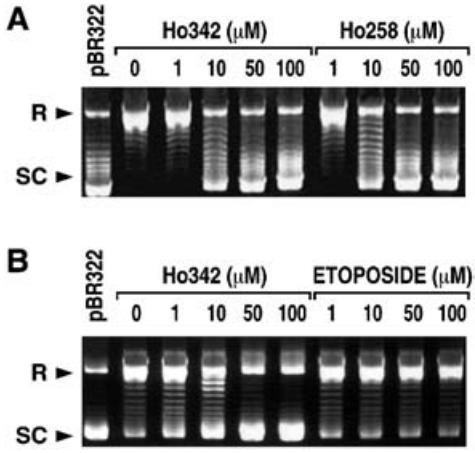

Fig. 3. Inhibition of Topo-I activity in *Leishmania donovani* extracts by Hoechst compounds. (A) Supercoiled pBR322 DNA alone in shown in lane 1. Supercoiled pBR322 DNA in the presence of parasite extracts and increasing concentrations of Ho342 and Ho258 (0–100 μM) are represented in the following lanes. R (Relaxed DNA), SC (Supercoiled DNA). (B) Supercoiled pBR322 DNA alone is shown in lane 1. Supercoiled pBR322 DNA in the presence of parasite extracts and increasing concentrations of Ho342 and etoposide (0–100 μM) are represented in the following lanes. R (Relaxed DNA), SC (Supercoiled DNA).

agarose gel containing ethidium bromide. The gel was run for 2·5 h at 50 V. The data presented represent 1 out of 3 independent experiments.

Statistical analysis

The results were analysed using the StatView™ SE + *Graphics* program. All statistical results (mean ± S.E.M.) were evaluated by the ANOVA test and were significant at 95%.

RESULTS

DNA MGBLs inhibit parasite growth

To test whether MGBLs inhibit parasites, we exposed freshly harvested *L. donovani* strain 1S2D, *L. mexicana mexicana*, *L. major* Friedlin, *L. braziliensis panamensis* promastigotes, and *T. brucei* trypomastigotes to Ho342 and Ho258. As shown in Fig. 1, in all cases except for the *L. braziliensis* culture, Ho342 inhibited parasite growth in a dose- and time-dependent manner. Maximal growth inhibition was attained at doses ranging from 10 to 25 μM for all strains of parasite tested. In contrast to Ho342, Ho258 did not inhibit the growth of these parasites, with the exception of *T. brucei* at doses of 25 to 100 μM. Since the majority of kinetoplastids used in this set of experiments were responsive to Ho342 treatment, *L. donovani* promastigotes were selected to delineate further the mechanisms by which MGBLs control kinetoplastid growth.

Hoechst 33342 nuclear localization in L. donovani *promastigotes*

To correlate the inhibition of parasite growth with possible nuclear accumulation of Ho342, *L. donovani* promastigotes were treated with various concentrations of Ho342 and Ho258. As reported in Fig. 2, we observed that the nuclear drug accumulation was dose dependent in the case of Ho342-treated over untreated parasites. In contrast, Ho258 accumulation

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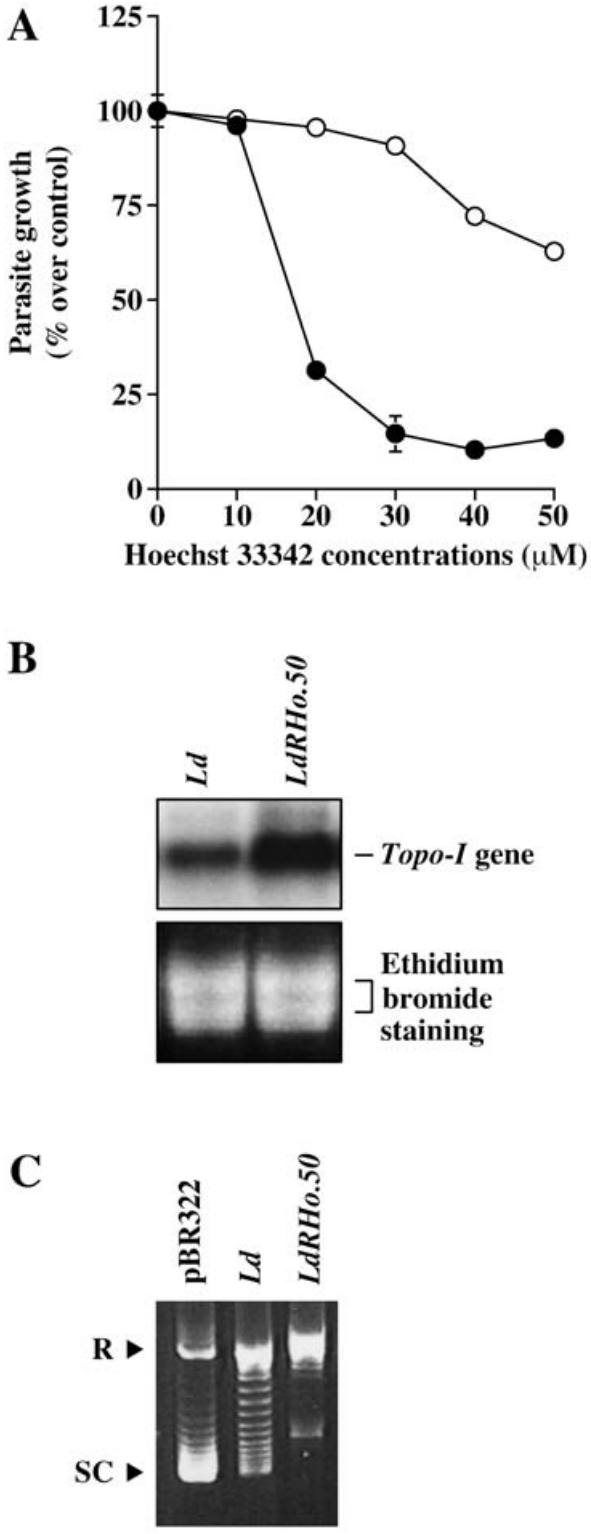

Fig. 4. Increase of Topo-I gene expression in Ho342-resistant *Leishmania donovani*. (A) *L. donovani* wild-type (●), and *L. donovani* resistant to Ho342 at 50 μM (○) were grown in the presence of increasing concentrations of Ho342 (0–50 μM). Parasite viability was measured by optical density after 6 days (mean ± S.E.M.). (B) Northern blots of *L. donovani* wild-type (*Ld*) and *L. donovani* resistant to Ho342 at 50 μM (*LdRHo.50*) with Topo-I probe. The probe used corresponds to the *Sac*I DNA fragment (1069 bp) from the *TOP1*-like gene of *L. donovani*. RNA integrity was verified by ethidium bromide staining. (C) DNA relaxation activity of *L. donovani* wild-type (*Ld*) and *L. donovani* resistant to Ho342 at 50 μM (*LdRHo.50*). Supercoiled pBR322 DNA alone is shown in lane 1. Supercoiled pBR322 DNA in the presence of both parasite extracts are represented in the following lanes. R (Relaxed DNA), SC (Supercoiled DNA).

in the parasite nucleus was not significantly detectable. Similar results have been obtained using total parasites (data not shown).

Hoechst compounds inhibit Topo-I activity in parasite extracts

The inhibitory effect of Ho342 and Ho258 on the activity of *L. donovani* Topo-I was evaluated on supercoiled pBR322 DNA, and revealed that both compounds were able to inhibit the Topo-I activity in parasite extracts (Fig. 3A). Hoechst compounds inhibited the relaxation activity of Topo-I in a dose-dependent manner, with maximal inhibition at 50 μM. Moreover, as reported in Fig. 3B, we demonstrated that etoposide, a Topo-II inhibitor, was unable to inhibit *L. donovani* Topo-I activity in parasite extracts, even at the highest concentration (100 μM) known to affect trypanosome Topo-II (Shapiro & Englund, 1990).

Increase of Topo-I gene expression in Hoechst 33342 resistant L. donovani

To support our observation concerning the specificity of Ho342 on *L. donovani* Topo-I, further we selected for parasites resistant to this compound. To reinforce our hypothesis, 3 resistant strains were developed from 3 different clones of wild-type parasite. Since each clone produced similar results, we have only presented 1 out of 3 parasite strains. *L. donovani* wild-type and 50 μM Ho342-resistant promastigotes were treated with increasing concentrations of Ho342 (0–50 μM). As shown in Fig. 4A, we observed a decrease in susceptibility to all doses of Ho342 in *L. donovani*-resistant strain, while the wild-type strain viability was drastically reduced in a dose-dependent manner. This difference in drug-sensitivity was clearly accompanied by an increase in Topo-I gene expression in resistant parasites, as reported in Fig. 4B. We also demonstrated that Topo-I gene expression was increased in a dose-dependent manner, and appeared to be significant starting from a resistance dose of 20 μM (data not shown), at which concentration we observed the first major difference in drug sensitivity (Fig. 4A). Moreover, the augmentation of Topo-I gene expression in the resistant strains was also corroborated by a higher Topo-I activity in these parasites (Fig. 4C). In the following

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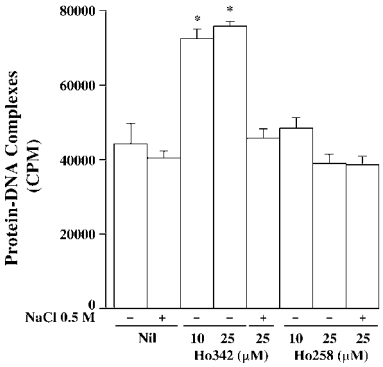

Fig. 5. Ho342-induced *Leishmania donovani* DNA break and reversion of cleavable complexes by NaCl. Radioisotope labelled parasites were treated with increasing concentrations of Ho342 and Ho258 (0–25 μM, 30 min), and DNA break measured by K^+-SDS assay (mean ± S.E.M.). Control group (Nil) represents the endogenous cleavable complexes formed in the absence of inhibitor. Reversion was performed using 25 μM treatments with NaCl 0·5 M before the lysing step. *Significant differences ($P < 0{\cdot}05$).

sections, we performed experiments to establish whether or not the Ho342 inhibitory effects toward *Leishmania* involved DNA damage.

Hoechst 33342 promotes formation of Topo-I-linked DNA breaks in L. donovani *promastigotes*

K^+-SDS assays were performed to determine whether or not Ho342 and Ho258 can form stable cleavable complexes between nuclear DNA and Topo-I in *L. donovani* promastigotes. As described above, [^{3}H]thymidine-labelled parasites were treated in the presence or absence of Hoechst compounds and then K^+-SDS assays were performed. As shown in Fig. 5, we observed an increase by more than 60% in the amount of DNA breaks when the parasites were treated with 10 and 25 μM of Ho342 as compared to untreated parasites. In addition, our study revealed that Ho258-treated *Leishmania* did not form cleavable complexes. Moreover, the formation of Topo-I-linked DNA breaks must be accompanied by the formation of reversible Topo-I-cleavable complexes. To test this possibility, 0·5 M NaCl was used to reverse Ho342-induced DNA breaks. As shown in Fig. 5, DNA fragmentation due to Ho342 at 25 μM was completely reverted by 0·5 M NaCl treatment. Moreover, Topo-I-linked DNA breaks induced by Ho342 correlated well with the concentrations of drug cytotoxicity.

Hoechst 33342-induced L. donovani *replication arrest occurs during the G_1 phase of the cell cycle*

In eukaryotes, classification of cellular division is generally divided into different cell cycle phases that can be recognized by their DNA contents. To

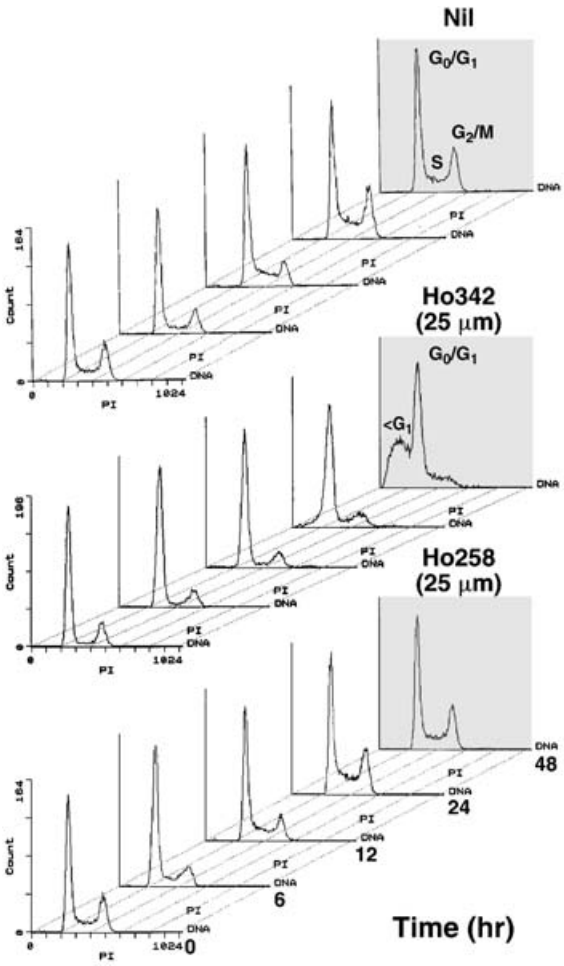

Fig. 6. Effects of Topo-I inhibitors on parasite cell cycle. *Leishmania donovani* promastigotes were treated or not with 25 μM of Ho342 and Ho258 over a 48-h period and cell cycle analysis performed as described in the Materials and Methods section. The cell populations corresponding to the different cell cycle phases (G_0/G_1, S, G_2M) and (< G_1) are represented in the top square of control group (Nil) and Ho342-treated parasites group (Ho342–25 μM) respectively.

pinpoint at which phase of the cell cycle Ho342 was acting upon parasite replication, *Leishmania* promastigotes were exposed to Ho342 and Ho258, and analysed by flow cytometry. As shown in Fig. 6, the replication of Ho342-treated parasites, as revealed by the sharp modification of the cell cycle pattern, was significantly blocked by a dose of 25 μM in comparison with the control. On the other hand, a lower concentration of Ho342 (10 μM) seemed to delay promastigote replication (data not shown). Importantly, Ho258-treated parasites showed normal cell cycle patterns, comparable to untreated *Leishmania*, an observation further reinforcing the correlation between parasite growth and normal processes of replication. In addition, our results clearly revealed that the G_1 population remained constant at 25 μM of Ho342 until the 24 h time-point in comparison with the controls. Moreover, when we analysed these same

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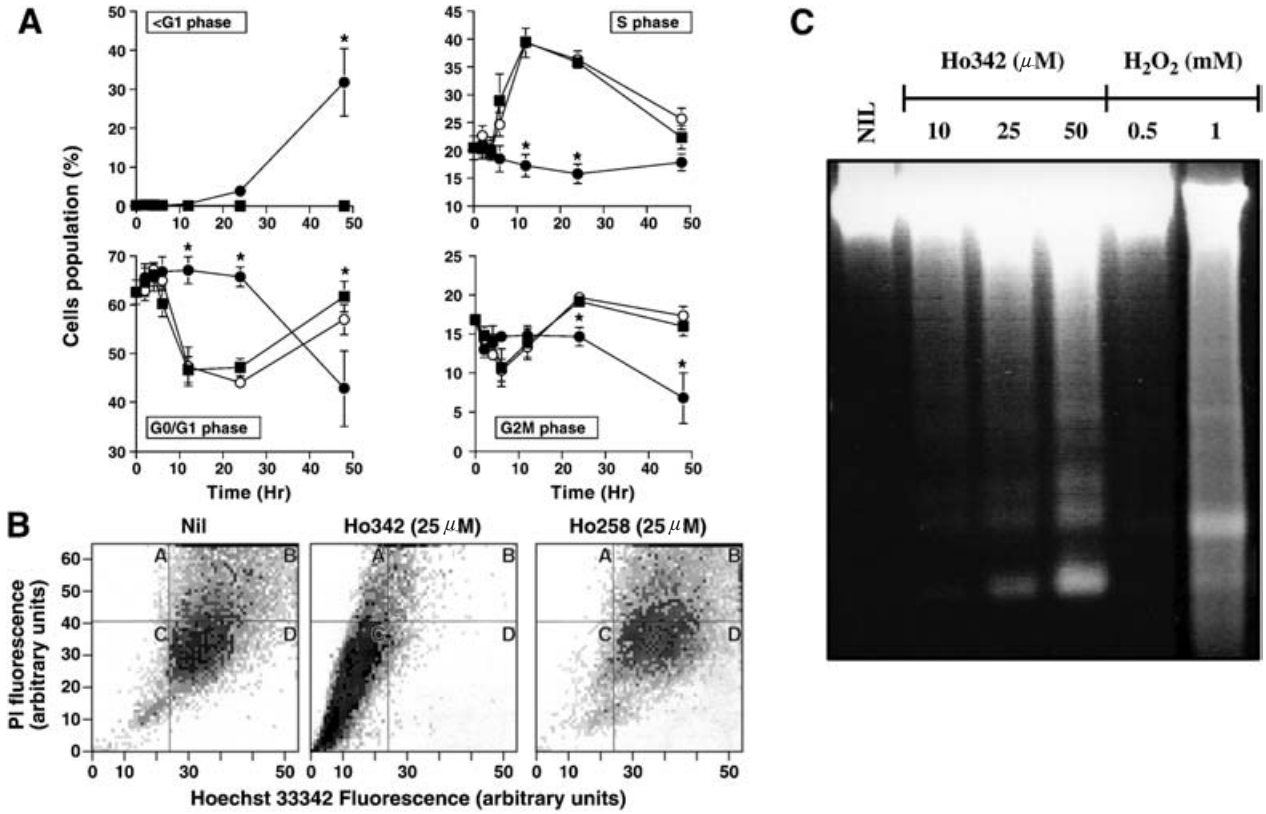

Fig. 7. Correlation between parasite death and apoptosis. (A) Cell cycle phase distribution of parasites treated or not with Ho342 and Ho258 (25 μM) over a 48-h period (mean $\pm$ S.E.M.). *Significant differences ($P<0{\cdot}05$). Nil (■); 25 μM Ho342 (●); 25 μM Ho258 (○). (B) Parasites were treated or not with 25 μM of Ho342 or Ho258 for 48 h, and prepared as described in the Materials and Methods section for apoptosis evaluation. Zone A (necrotic cells), zone B (S/G_2M phase), zone C (apoptotic cells), and zone D (G_0/G_1 phase). (C) Pattern of *L. donovani* DNA fragmentation on agarose gel following Ho342 (0–50 μM) and H_2O_2 (0·5 and 1 mM) treatments for 48 h.

results in a phase-specific manner (see Fig. 7A); after 48 h of treatment with Ho342 at 25 μM, a significant decrease (more than 22%) of the percentage of cells in the G_1 phase was observed, together with a concomitant increase in cells in $<G_1$ phase (more than 31% of total cells). These results suggest that the replication of Ho342-treated *Leishmania* was blocked in G_1 phase. As the population prior to the G_1 phase displayed DNA fragmentation typical of apoptosis, it was important to verify whether this arrest of parasite growth and subsequent death involved an apoptotic-like phenomenon. To accomplish this, parasites were treated with either Ho342 or Ho258 (25 μM), and the percentages of apoptotic cells were determined. As reported in Fig. 7B, we determined that $81{\cdot}15 \pm 1{\cdot}55$% of the Ho342-treated parasites were in the apoptotic zone in comparison with the Ho258-treated group ($9{\cdot}20 \pm 3{\cdot}20$%) and the untreated control ($12{\cdot}95 \pm 7{\cdot}35$%). We also evaluated in parallel the phenomenon of DNA fragmentation on Ho342-treated parasites. *L. donovani* parasites were treated with Ho342 (10, 25 and 50 μM), and then DNA was extracted and analysed by electrophoresis. As is apparent from Fig. 7C, we observed a DNA fragmentation pattern when parasites were treated with increasing doses of Ho342. Moreover, since H_2O_2 was recognized to induce apoptotic-like events in *L. donovani* (Das, Mukherjee & Shaha, 2001), as a control we compared its capability to fragment parasite DNA. As reported in Fig. 7C, if we compare 50 μM Ho342- and 1 mM H_2O_2-treated parasites, both compounds seem to induce similar patterns of DNA fragmentation.

DISCUSSION

It is now well established that Topo-I plays a pivotal role in the regulation of DNA functions (Wang, 1985, 1991). The development of drugs that target nuclear Topo-I could represent an important avenue for the development of new therapeutic agents against the *Leishmania* parasite (Burri, Bodley & Shapiro, 1996).

In the present study, we obtained clear evidence that, in contrast to Ho258, Ho342 completely blocked parasite replication in a dose- and time-dependent manner. Our results are in agreement with those reported for the impact of Ho342 treatment on calf thymus Topo-I (Chen *et al.* 1993*b*), human KB cells (Chen *et al.* 1993*a*), and camptothecin-resistant human lymphoblast CPT-K5 cells (Chen *et al.* 1993*a*). Surprisingly, Ho258 seems to be ineffective on *Leishmania* spp., contrary to the effect previously observed

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on mammalian DNA Topo-I (Chen *et al*. 1993*b*). Our results on parasite growth show that Ho342 cytotoxicity against *L. donovani* was obtained at a similar ED50 as camptothecin treatment on the same pathogen (Bodley & Shapiro, 1995). Thereafter, Ho342 parasite growth inhibition observed in our study was correlated with the capacity of this compound to enter the cell and reach the nuclear compartment of *L. donovani*, in contrast to Ho258. The greater efficacy of Ho342 might be explained by its higher lipophilicity, one of the determining factors of passive diffusion across the cellular membrane (Chen *et al*. 1993*a*). This could be due to the ethoxy substitution on the 4-phenyl ring of its parent compound Ho258 (Chen *et al*. 1993*a*). Relaxation activity assays reveal the toxicity of both Hoechst compounds toward the Topo-I activity of *L. donovani* supporting the fact that Ho342 cytotoxicity on parasite growth, compared to Ho258, is due to a difference in cellular permeability. Moreover, this compound's effect on *L. donovani* Topo-I activity was reinforced by the development of resistance to Ho342 that seems to be conferred by an increase in Topo-I gene expression and a higher level of Topo-I activity. However, in general, the resistance to Topo-I inhibitors in mammalian cells is multifactorial and involves mechanisms associated with drug transport (Endicott & Ling, 1989), drug–target interaction (Beck, 1987) and drug detoxification (Deffie *et al*. 1988). Until now, resistance of *L. donovani* to Ho342 seems to be related to drug–target interaction, but additional experiments are needed to verify this hypothesis. The inhibiting ability of Ho342 toward the Topo-I activity of *L. donovani* is also consistent with the higher level of protein–DNA complexes *in vitro*, which reflect Topo-I-cleavable complexes, in Ho342-treated *L. donovani*. A similar formation of nuclear protein–DNA complexes was observed in camptothecin-treated Trypanosomatidae (Bodley & Shapiro, 1995). As previously reported (Chen *et al*. 1993*b*), the reversal of these protein–DNA complexes by NaCl treatment provides additional evidence for the Ho342 Topo-I enzyme toxicity, since such NaCl-mediated reversions are well recognized as a hallmark of Topo-I-cleavable complexes.

Our experiment designed to show the cell cycle phase at which the Ho342 acts, showed a G_1 phase specificity for treated *L. donovani*, in contrast with the G_2M arrest reported for Chinese hamster cells V79 (Durand & Olive, 1982). Moreover, the decrease of parasites in the G_1 phase was concomitant with the appearance of a pre-G_1 phase population as a late event. This observation was of interest since this population of $< G_1$ phase corresponds with the DNA fragmentation that could have resulted from programmed cell death, as previously observed in cancer cells subjected to anti-topoisomerase treatment (Nitiss & Beck, 1996), in *L. donovani* subjected to H_2O_2 treatment (Das *et al*. 2001) and in Sb(III) antimonial-treated *L. infantum* (Sereno *et al*. 2001). Moreover, recent studies have also specifically demonstrated that Ho342 and not Ho258 induces apoptosis in BC3H-1 myocytes and hepatoma cells (Zhang & Kiechle, 1997, 1998*a*, *b*). Our observation was supported by a double staining method that discriminates between apoptosis and necrosis, and also by DNA fragmentation on agarose gel in *L. donovani* treated with Ho342. In the light of these findings, we hypothesized that the pathway used by Ho342 to induce DNA damage triggered cellular events in *L. donovani* leading to cell death by a mechanism that closely resembles apoptosis.

This study reveals a Topo-I effect of Ho342 toward the *Leishmania* parasite, in respect to its ability to enter the parasite nucleus and to interact with this enzyme. Although Ho342 is also effective on mammalian cells, the main goal of our investigations was to demonstrate its specificity for Topo-I in a *Leishmania* model and to reveal the potential of the lead molecule (2,5′-Bi-1*H*-benzimidazole) as a starting structure for derivative compounds as being more effective. As previously reported, the structure-activity of Topo-I poisons related to Ho342 has already been evaluated in a diverse type of cancer cells (Sun *et al*. 1994). These structural variations did not affect the capacity of the resulting Ho342 derivatives to act as Topo-I inhibitors. Therefore, MGBLs, including Ho342, represent a class of promising Topo-I inhibitors that could potentially be used as therapeutic agents against *Leishmania* infections.

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