



2-Alkynoic fatty acids inhibit topoisomerase IB from *Leishmania donovani*

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ABSTRACT

2-Alkynoic fatty acids display antimycobacterial, antifungal, and pesticidal activities but their antiprotozoal activity has received little attention. In this work we synthesized the 2-octadecynoic acid (2-ODA), 2-hexadecynoic acid (2-HDA), and 2-tetradecynoic acid (2-TDA) and show that 2-ODA is the best inhibitor of the *Leishmania donovani* DNA topoisomerase IB enzyme (*LdTopIB*) with an $EC_{50} = 5.3 \pm 0.7 \mu\text{M}$. The potency of *LdTopIB* inhibition follows the trend 2-ODA > 2-HDA > 2-TDA, indicating that the effectiveness of inhibition depends on the fatty acid carbon chain length. All of the studied 2-alkynoic fatty acids were less potent inhibitors of the human topoisomerase IB enzyme (*hTopIB*) as compared to *LdTopIB*. 2-ODA also displayed in vitro activity against *Leishmania donovani* ($IC_{50} = 11.0 \mu\text{M}$), but it was less effective against other protozoa, *Trypanosoma cruzi* ($IC_{50} = 48.1 \mu\text{M}$) and *Trypanosoma brucei rhodesiense* ($IC_{50} = 64.5 \mu\text{M}$). The antiprotozoal activity of the 2-alkynoic fatty acids, in general, followed the trend 2-ODA > 2-HDA > 2-TDA. The experimental information gathered so far indicates that 2-ODA is a promising antileishmanial compound.

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Acetylenic fatty acids have entertained both medicinal chemists and natural products chemists for the diversity of biological activities that they have displayed.^{1,2} Among these fatty acids the octadecynoic acids are worth mentioning. Earlier studies identified the 5-octadecynoic acid (5-ODA) as a natural product from the root of the plant *Ximena americana* with pesticidal activity as demonstrated by the inhibition of the hatching of the pod-sucking bug *Clavigralla tomentosicollis* eggs.³ On the other hand, the roots of *Pentagonia gigantifolia* yielded the acid 6-octadecynoic acid (6-ODA), which displayed potent antifungal activity against a series of fluconazole resistant *Candida albicans* strains with IC_{50} values between 0.45–0.65 $\mu\text{g/mL}$.⁴ Among a series of 2-alkynoic fatty acids the 2-octadecynoic acid (2-ODA) and its metabolites displayed the best antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG by inhibiting fatty acid biosynthetic pathways of considerable importance for mycobacteria.⁵ Interesting to mention is that by placing the unsaturation at the other extreme of the acyl chain, the acid 17-octadecynoic acid (17-ODA) is obtained, which is a well-known suicide inhibitor of the leukotriene B_4 ω -oxidase ($IC_{50} < 5 \mu\text{M}$) as well as a selective inhibitor of the renal CYP450 ω -hydroxylase.⁶ All of these findings attest to the biological potential of the octadecynoic acids as anti-infective agents and enzyme inhibitors, but no data on the inhibi-

tion of key enzymes in *Leishmania* parasites by these acids has been reported.

Several other 2-alkynoic fatty acids show antiprotozoal activity and inhibit protozoal enzymes. For example, our team studied the antiprotozoal activity of 2-hexadecynoic acid (2-HDA) and found that it effectively inhibited plasmodial FAS-II enzymes (IC_{50} 's between 1.5 and 13.9 μM) and arrests erythrocytic and liver stage *Plasmodium* infections.⁷ In addition, we showed that 2-HDA displays antiprotozoal activity against *Leishmania donovani* amastigotes ($IC_{50} = 17.8 \mu\text{M}$) but no studies on key *L. donovani* enzymes amenable for therapeutic intervention were performed. These initial results motivated us to study 2-TDA, 2-HDA and 2-ODA as antiprotozoal compounds so as to determine the antiprotozoal activity of the acids as a function of carbon chain length. Therefore, in this work we synthesized 2-TDA, 2-HDA, and 2-ODA and determined their growth inhibitory activities against *L. donovani*, *T. cruzi*, and *Trypanosoma brucei rhodesiense* in vitro. The best antiprotozoal results were obtained against *L. donovani* and therefore, we studied the inhibition of the *LdTopIB* enzyme by 2-TDA, 2-HDA, and 2-ODA and compared it to *hTopIB*.

DNA topoisomerases have been the target of many studies since early successes with the camptothecins and similar structural analogs.⁸ In particular, type I DNA topoisomerases are a well-recognized target for cancer therapy. The trypanosomal and leishmania type IB DNA topoisomerases differ significantly from the homologous mammalian structures since they are phylogenetically

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unique and possess an anomalous dimeric structure.^{9,10} These enzymes have been recognized as excellent targets for the development of antiparasitic drugs. However, no clear structure activity relationships (SAR) exist for the interaction of drugs, in particular fatty acids, against these type IB topoisomerases. Our series of 2-alkynoic fatty acids give us an excellent tool to gain better insights into the structural requirements needed for effective *LdTopIB* inhibition by fatty acids, in particular if these activities can be correlated to the toxicity against *L. donovani*. The results of this study indicate that C₁₈ is an effective carbon chain length among the 2-alkynoic acids for inhibiting *LdTopIB*, which happens to correlate with the toxicities observed against *L. donovani* axenic amastigotes.

The synthesis of 2-TDA, 2-HDA, and 2-ODA followed an already published procedure⁷ wherein commercially available 1-tridecyne, 1-pentadecyne, or 1-heptadecyne was reacted with *n*-BuLi in THF at -70 °C followed by quenching with CO₂ and final protonation with NH₄Cl (Fig. 1). The yields ranged from 45–79%. The purity of the synthesized compounds was determined to be >95% by capillary GC-MS and ¹³C NMR. The spectral data of the synthesized 2-alkynoic fatty acids were in agreement with those previously reported.^{5,7}

The antiprotozoal activities of the synthesized 2-alkynoic fatty acids were studied against *Leishmania donovani* (axenic amastigotes), *Trypanosoma brucei rhodesiense* (bloodstream forms), and *Trypanosoma cruzi* (intracellular amastigotes in L6 rat skeletal myoblasts) as previously described.⁷ As shown in Table 1, among the 2-alkynoic fatty acids 2-ODA displayed the best antiprotozoal activity against all the studied protozoa with IC₅₀ values ranging between 11.0 and 64.5 μM, followed by 2-HDA (IC₅₀s between 17.8 and 83.6 μM) and finally 2-TDA (IC₅₀s between 24.7 and 255.4 μM). Therefore, the 2-acetylenic fatty acids were effective in killing the protozoa by following the order 2-ODA > 2-HDA > 2-TDA. *Leishmania donovani* amastigotes were the most susceptible to the 2-alkynoic fatty acids (IC₅₀s between 11.0 and 24.7 μM), but the test compounds were not as effective against *T. cruzi* (IC₅₀s between 62.4 and 80.0 μM) and *T. brucei rhodesiense* (IC₅₀s between 64.5 and 255.4 μM). Overall, 2-ODA showed the broadest spectrum of antiprotozoal activity, but the most effective effect was observed against *L. donovani*.

Aimed at exploring a possible mechanism responsible for the antileishmanial activity displayed by the 2-alkynoic fatty acids apoptosis was probed in *Leishmania infantum* (2-HDA displayed an IC₅₀ of 14.9 μM against *L. infantum* promastigotes) by detecting the translocation of phosphatidylserine (PS) to the cell surface with the Annexin-FITC reagent. To test for apoptosis in *Leishmania* is quite reasonable since it has been reported that *Leishmania* amastigotes can fake its own death by exposing PS on its surface and gain access to macrophages.¹¹ This PS translocation results in the inhibition of NO production and the induction of TGF-β secretion and IL-10 synthesis.¹¹ In our experiment (Fig. 2) *L. infantum* promastigotes were treated with 2-TDA, 2-HDA, and 2-ODA at concentrations ranging from 100 to 400 μM. At the highest concentration of 400 μM (Fig. 2) no significant concentration of apoptotic cells were observed for neither of the acetylenic fatty acids tested, but a positive test for hydrogen peroxide as the control was observed.¹² This experiment demonstrates that apoptosis is not the

main mechanism by which the 2-alkynoic acids kill the *Leishmania* promastigotes.

Other mechanisms of toxicity for 2-TDA, 2-HDA, and 2-ODA in *L. donovani* were examined by concentrating on the inhibition of the *Leishmania donovani* topoisomerase IB enzyme (*LdTopIB*) (Table 2, Fig. 3). One of the most recent contributions against protozoan-caused infectious diseases takes advantage of the structural differences between the protozoan and the host TopIB, since the unusual heterodimeric TopIB of kinetoplastid parasites, such as *LdTopIB*, can be used for the development of new compounds targeting only the parasite TopIB without interfering with the monomeric TopIB of the human host.¹³ For this reason, the inhibition of *LdTopIB* by the 2-alkynoic fatty acids was examined and compared to *hTopIB*. As expected, 2-ODA was the most efficient inhibitor with an EC₅₀ = 5.3 ± 0.7 μM followed by 2-HDA with an EC₅₀ = 28.7 ± 1.3 μM (Fig. 3). The effectiveness of inhibition followed the order 2-ODA > 2-HDA > 2-TDA. This trend correlates quite well with the toxicity displayed by the 2-alkynoic acids towards the *L. donovani* amastigotes.

In the latter experiment the inhibition of 2-ODA towards *hTopIB* was compared to *LdTopIB* and the results are also shown in Table 2 and Figure 3. While 2-ODA was able to inhibit *LdTopIB* at 5.3 μM, it was less effective against *hTopIB* (EC₅₀ = 51.9 μM). In fact, neither 2-TDA nor 2-HDA was inhibitory towards *hTopIB* at 100 μM. These results clearly reveal that it will be possible to preferentially interfere with *LdTopIB* without inhibiting the human enzyme, a finding that could have therapeutic value. It is evident that *LdTopIB* is more sensitive to fatty acid inhibition than *hTopIB*.

The next study contemplated if the 2-alkynoic fatty acids inhibited *hTopIB* and *LdTopIB* with a similar or different mechanism as camptothecin (CPT), a well-known topoisomerase I inhibitor.⁸ Although, 2-HDA is a bit less inhibitory than 2-ODA towards *LdTopIB*, the availability of compound prompted us to choose 2-HDA as the model fatty acid to study the fatty acid-CPT mechanism. From Figure 4 it is evident that 2-HDA inhibits the catalytic activity of *LdTopIB* at 100 μM concentration (DNA is supercoiled in the presence of the acid) but there is no inhibition of *hTopIB* since the DNA substrate is totally relaxed at the same 2-HDA concentration. However, from Figure 4a (lane 5 under human) it seems that 2-HDA enhances the stabilization of cleavable complexes between CPT and *hTopIB* and prevents the formation of the corresponding cleavable complex made between CPT and *LdTopIB* (Fig. 4a, lane 5 under *Leishmania*). In order to further explore the mechanism of 2-HDA inhibition two parallel experiments were then performed. In a control experiment *LdTopIB* was first reacted with the supercoiled plasmid DNA and CPT in DMSO for 15 min at 4 °C and in another experiment *LdTopIB* was pre-incubated (15 min) first with 2-HDA (100 μM) followed by the addition of 100 μM CPT and the experiment was run for the same amount of time as the control. The left panel in Figure 4b shows that CPT inhibited *LdTopIB* in a reversible way when it was pre-incubated with DMSO (control), and after 4 min the supercoiled band was less intense and the relaxed DNA band became stronger. In addition, the nicked DNA band in Figure 4b (left) grew in intensity due to the stabilization of the ternary cleavage complexes (poisoning effect of CPT). The right panel of Figure 4b shows an assay where a pre-incubation of 2-HDA and *LdTopIB* was performed before the addition of DNA and CPT. Two interesting findings were obtained: i) there was no relaxation of the DNA at any extent, since only the supercoiled band was present when the gel was run, and ii) the nicked band did not grow in intensity with respect to the control lane, so CPT was unable to stabilize the cleavable complexes. If the enzyme were to cut the DNA substrate, CPT could interact with the DNA-protein complexes and thus stabilize them. Therefore, we can hypothesize that 2-HDA inhibits the *LdTopIB*-mediated DNA relaxation by a complete different mechanism as CPT, and prevents the formation of cleavable

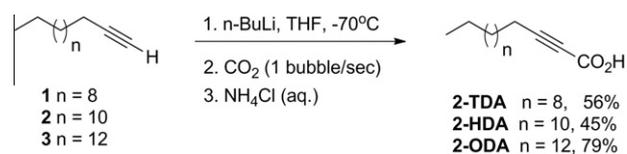


Figure 1. Synthesis of 2-TDA, 2-HDA, and 2-ODA with the corresponding yields.

Table 1
Antiprotozoal activities of the 2-alkynoic fatty acids. The IC₅₀ values are in μM.

Compounds	<i>Trypanosoma brucei rhodesiense</i>	<i>Trypanosoma cruzi</i>	<i>Leishmania donovani</i>	L6 cell cytotoxicity
2-TDA	255.4	62.4	24.7	40.9
2-HDA	83.6	80.0	17.8	340.3
2-ODA	64.5	48.1	11.0	20.0
Standard	0.0075 ^a	1.47 ^b	0.49 ^c	0.02 ^d

The values represent the average of at least two independent assays performed in duplicates. Reference compounds are: ^aMelarsoprol, ^bBenznidazole, ^cMiltefosine, ^dPodophyllotoxin.

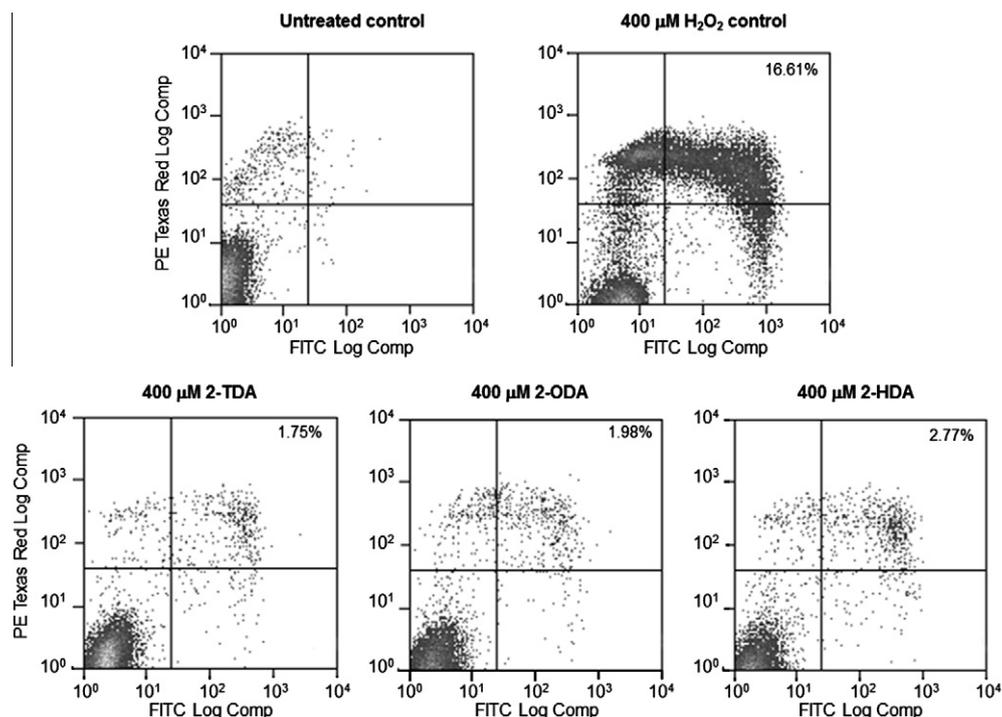


Figure 2. Apoptosis analysis by staining with Annexin-V-FITC and propidium iodide. Untreated and H₂O₂ treated cell were used as negative and positive controls, respectively. Top right quadrant shows the percent of late apoptotic cells. Apoptosis was detected by translocation of phosphatidyl serine (PS) to the cell surface with the annexin V-FITC reagent (BD Pharmingen). Fraction of Annexin V-positive cells was measured with CellQuest software (BD Biosciences, San Jose, CA). To estimate the number of apoptotic cells *L. infantum* promastigotes was treated with 400 μM compounds for 24 h, harvest, wash twice with PBS, and resuspended in 1 × 10⁶ cells/ml. Cells were fluorescently labeled by addition of 20 μl of binding buffer, 5 μl of Annexin V-FITC and 5 μl of propidium iodide. After incubation at room temperature in the dark for 15 min, cells were applied to flow cytometry analysis. A minimum of 10,000 cells in the gated region was analyzed using a BD FACS Calibur Flow Cytometer. Results were interpreted by the percentage of total cells appearing in each quadrant. The experiment is representative of three independent trials.

Table 2
Inhibition of the relaxation activities of the *LdTopIB* and *hTopIB* enzymes by the studied fatty acids and their toxicities against murine macrophages (μM)

Compounds	<i>LdTopIB</i> EC ₅₀	<i>hTopIB</i> EC ₅₀	Murine macrophages BALB/c IC ₅₀
2-TDA	67.8 ± 1.1	>100	>100
2-HDA	28.7 ± 1.3	>100	>100
2-ODA	5.3 ± 0.7	51.9 ± 1.0	>100
CPT	0.67 ± 0.08	2.0 ± 1.0	0.62 ± 0.13

complexes avoiding the ability of CPT to stabilize enzyme-DNA complexes. This is consistent with the findings of Castelli and co-workers where they analyzed the effect of conjugated eicosapentaenoic acid (cEPA) on the homologous human enzyme catalytic cycle.¹⁴ In particular, pre-incubation of cEPA with *hTopIB* before addition of DNA did not permit the stabilization of the cleavable complex by CPT and, in addition, cleavage inhibition was enhanced.¹⁴ Additional experiments with a suicide substrate, where the enzyme is not able to carry out the religation step, revealed an identical religation rate with or without the presence of cEPA. This is in contrast to what is

normally observed with CPT, which strongly reduces the topoisomerase IB religation rate.¹⁴ Taken as a whole, the most plausible explanation in our system is for the 2-alkynoic fatty acids to be interacting with *LdTopIB* by binding in a region close to the topoisomerase active site and either inhibiting the enzyme binding to DNA or blocking the cleavage reaction step.

Toxicity of the 2-alkynoic fatty acids was determined against L6 cells, a primary cell line from rat skeletal myoblasts. As shown in Table 1 the 2-ODA was the most toxic of the three 2-alkynoic fatty acids towards L6 cells (IC₅₀ = 20 μM). Surprisingly, 2-HDA displayed negligible toxicity towards L6 cells (IC₅₀ = 340.3 μM), which makes it a more therapeutically valuable compound. While 2-HDA seems to be an outlier in Table 1 we also determined that palmitic acid displays an IC₅₀ of 209 μM against these L6 cells. This marked difference between the toxicity of fatty acids towards L6 cells does not seem to be novel. For example, oleate and palmitate display different effects with respect to mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells.¹⁵ Selective toxicity of the 2-alkynoic acids was also studied against BALB/c murine macrophages (Table 2). In this system, none of the 2-alkynoic fatty acids displayed significant toxicity, that is, in all three examples

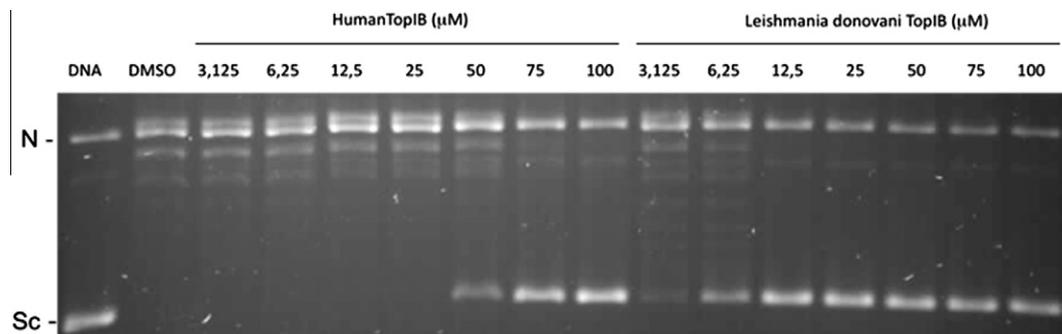


Figure 3. Comparison of the inhibition of the relaxation activity of human TopIB (left) and recombinant *LdTopIB* (right) by 2-ODA. One unit of recombinant *LdTopIB* was assayed in a plasmid DNA relaxation assay for 30 min at 37 °C in the presence of 3.125–100 μM 2-ODA. Reaction products were resolved in agarose gel and subsequently visualized by ethidium bromide staining. The relative position of the negatively supercoiled DNA substrate is indicated by Sc, N is the nicked DNA, whereas the ladder of relaxed DNA topoisomer bands is shown in between. Reactions were stopped with a mixture of 1% SDS and 6.1 μg of proteinase K. Lane 1 contains 0.5 μg of pSK plasmid DNA and lane 2 DMSO.

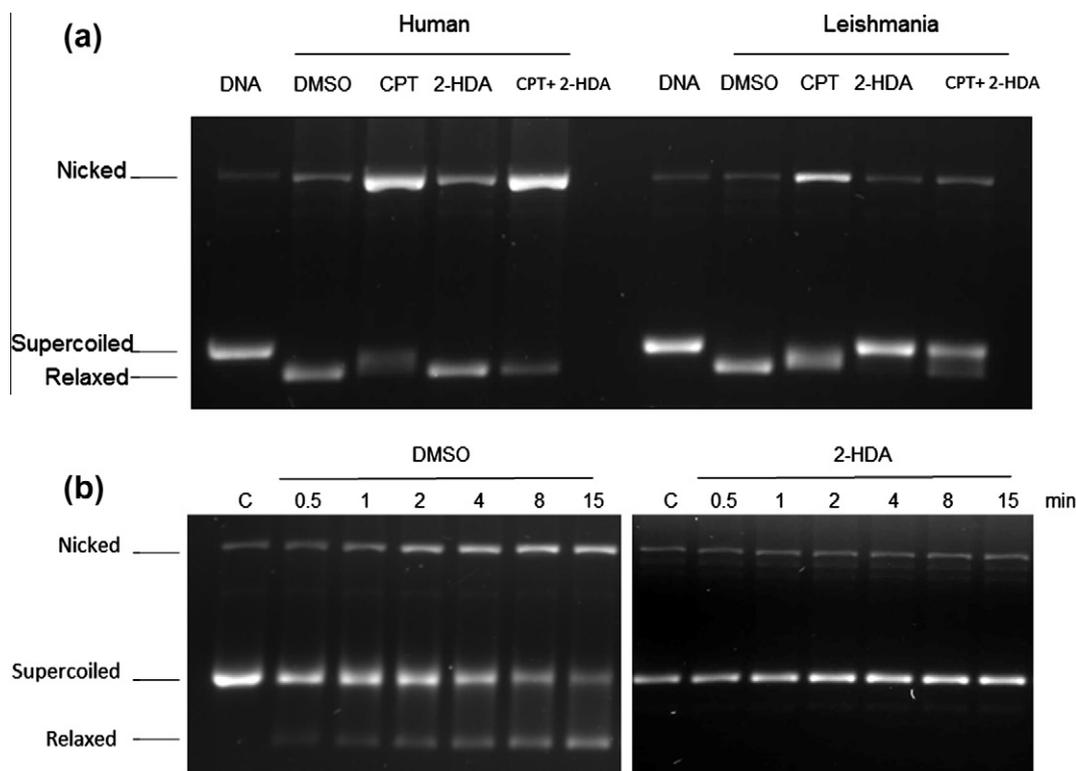


Figure 4. Supercoiled plasmid DNA TopIB-mediated cleavage assay comparing the inhibition and poisoning of recombinant *hTopIB* (A left) and *LdTopIB* (A right) by 2-HDA and CPT. Both enzymes were assayed in the presence of DMSO (drug vehicle), 100 μM CPT (positive control for cleavage stabilization), 100 μM 2-HDA and a mix of 100 μM 2-HDA/CPT. Samples were incubated for 4 min at 25 °C, stopped with 1% SDS and digested for one extra hour at 37 °C in the presence of 1 mg/ml proteinase K. DNA was extracted with one volume of phenol-chloroform and samples were run on a 1% agarose gel containing ethidium bromide to a final concentration of 40 μg/ml in order to separate supercoiled and relaxed DNA. The bottom figure (B left panel) shows that a pre-incubation (15 min at 4 °C) with DMSO had no influence on the CPT inhibiting/poisoning mechanism, as the nicked DNA band increased its relative intensity with respect to the control, at different time intervals. When *LdTopIB* was pre-incubated with a 2-HDA excess (100 μM) previous to the addition of 0.5 μg of pSK DNA the enzymatic activity was fully inhibited by 2-HDA and CPT poisoning effect did not take place (B right panel). After the time-course assay samples were extracted and run as previously described in 4A. The nicked band corresponds to the stabilized cleavage complexes. The results are representative of three independent trials.

the IC_{50} value was >100 μM. This result indicates that the 2-alkynoic fatty acids display different toxicity towards different cell lines. The difference in toxicity between BALB/c and L6 cells for 2-ODA could also lie in the fact that we used a primary cell line for the latter.

The results presented herein indicate that among the studied 2-alkynoic fatty acids 2-ODA is the most toxic against *L. donovani* amastigotes with a mechanism of action that could involve, although not exclusively, the inhibition of the *LdTopIB* enzyme. As to the reasons why 2-ODA is a better inhibitor than 2-HDA or

2-TDA we can only speculate at this point since there is no complete crystal structure for *LdTopIB* available, only partial structures of regions similar to *hTopIB* or computational simulations of the whole enzyme. However, the increased affinity of 2-ODA could be simply due to an increase number of van der Waals interactions with *LdTopIB* by the extra methylene groups in the molecule. More research is evidently needed in order to elucidate the subtle differences in the interaction of fatty acids with *LdTopIB* vs. *hTopIB*. However, as mentioned above, one possible mechanism of inhibition is for the 2-alkynoic fatty acids to interact with *LdTopIB* by

binding in a region close to the topoisomerase active site and just blocking the cleavage reaction, as it was demonstrated in the interaction of cEPA with *hTopIB*.¹⁴ In any instance, 2-ODA or 2-HDA stand out as useful fatty acids that could be used in drug formulations to treat leishmaniasis.

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