Bioorganic & Medicinal Chemistry Letters 22 (2012) 6185–6189

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

Néstor M. Carballeira [a,*], Michelle Cartagena [a], David Sanabria [b], Deniz Tasdemir [c], Christopher F. Prada [d], Rosa M. Reguera [d], Rafael Balaña-Fouce [d]

[a] Department of Chemistry, University of Puerto Rico, PO Box 23346, San Juan 00931-3346, Puerto Rico
[b] Department of Natural Sciences, Interamerican University, Metropolitan Campus, San Juan 00919, Puerto Rico
[c] School of Chemistry, National University of Ireland Galway, Galway, Ireland
[d] Department of Biomedical Sciences, University of León, Campus de Vegazana s/n, 24071 León, Spain

ARTICLE INFO

Article history:
Received 22 June 2012
Revised 20 July 2012
Accepted 1 August 2012
Available online 10 August 2012

Keywords:
Acetylenic fatty acids
Leishmania donovani
2-Octadecynoic acid
Topoisomerase IB

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 (*Ld*TopIB) with an $EC_{50} = 5.3 \pm 0.7$ μM. The potency of *Ld*TopIB 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 (*h*TopIB) as compared to *Ld*TopIB. 2-ODA also displayed in vitro activity against *Leishmania donovani* ($IC_{50} = 11.0$ μM), but it was less effective against other protozoa, *Trypanosoma cruzi* ($IC_{50} = 48.1$ μM) and *Trypanosoma brucei rhodesiense* ($IC_{50} = 64.5$ μM). 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.

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 μg/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 ($IC_{50} < 5$ μM) 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 inhibition 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 ($IC_{50} = 17.8$ μM) 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 *Ld*TopIB enzyme by 2-TDA, 2-HDA, and 2-ODA and compared it to *h*TopIB.

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

* Corresponding author.
E-mail address: nestor.carballeira1@upr.edu (N.M. Carballeira).

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http://dx.doi.org/10.1016/j.bmcl.2012.08.019

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 *Ld*TopIB 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 C_{18} is an effective carbon chain length among the 2-alkynoic acids for inhibiting *Ld*TopIB, 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 procedure[7] wherein commercially available 1-tridecyne, 1-pentadecyne, or 1-heptadecyne was reacted with *n*-BuLi in THF at -70 °C followed by quenching with CO_2 and final protonation with NH_4Cl (Fig. 1). The yields ranged from 45–79%. The purity of the synthesized compounds was determined to be >95% by capillary GC–MS and ^{13}C 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 IC_{50} values ranging between 11.0 and 64.5 μM, followed by 2-HDA ($IC_{50's}$ between 17.8 and 83.6 μM) and finally 2-TDA ($IC_{50's}$ 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 ($IC_{50's}$ between 11.0 and 24.7 μM), but the test compounds were not as effective against *T. cruzi* ($IC_{50's}$ between 62.4 and 80.0 μM) and *T. brucei rhodesiense* ($IC_{50's}$ 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 IC_{50} 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.

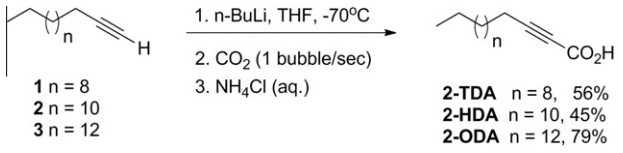

Figure 1. Synthesis of 2-TDA, 2-HDA, and 2-ODA with the corresponding yields.

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 (*Ld*TopIB) (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 *Ld*TopIB, 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 *Ld*TopIB by the 2-alkynoic fatty acids was examined and compared to *h*TopIB. As expected, 2-ODA was the most efficient inhibitor with an EC_{50} = 5.3 ± 0.7 μM followed by 2-HDA with an EC_{50} = 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 *h*TopIB was compared to *Ld*TopIB and the results are also shown in Table 2 and Figure 3. While 2-ODA was able to inhibit *Ld*TopIB at 5.3 μM, it was less effective against *h*TopIB (EC_{50} = 51.9 μM). In fact, neither 2-TDA nor 2-HDA was inhibitory towards *h*TopIB at 100 μM. These results clearly reveal that it will be possible to preferentially interfere with *Ld*TopIB without inhibiting the human enzyme, a finding that could have therapeutic value. It is evident that *Ld*TopIB is more sensitive to fatty acid inhibition than *h*TopIB.

The next study contemplated if the 2-alkynoic fatty acids inhibited *h*TopIB and *Ld*TopIB 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 *Ld*TopIB, 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 *Ld*TopIB at 100 μM concentration (DNA is supercoiled in the presence of the acid) but there is no inhibition of *h*TopIB 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 *h*TopIB and prevents the formation of the corresponding cleavable complex made between CPT and *Ld*TopIB (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 *Ld*TopIB was first reacted with the supercoiled plasmid DNA and CPT in DMSO for 15 min at 4 °C and in another experiment *Ld*TopIB 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 *Ld*TopIB 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 *Ld*TopIB 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 *Ld*TopIB-mediated DNA relaxation by a complete different mechanism as CPT, and prevents the formation of cleavable