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Recent developments in the antiprotozoal and anticancer activities of the 2-alkynoic fatty acids

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Abstract

The 2-alkynoic fatty acids are an interesting group of synthetic compounds that display antimycobacterial, antifungal, anticancer, and pesticidal activities but their antiprotozoal activity has received little attention until recently. In this review we have summarized our present knowledge of the biomedical potential of the 2-hexadecynoic acid (2-HDA) and 2-octadecynoic acid (2-ODA) together with several mechanistic pieces of work attesting to the fact that these compounds, and their metabolites, are good fatty acid biosynthesis inhibitors. The antiprotozoal activity of 2-HDA and 2-ODA against *Leishmania donovani* and *Plasmodium falciparum*, parasites responsible for visceral leishmaniasis and malaria, respectively, is also reviewed. The evidence obtained so far supports the fact that these fatty acids are good inhibitors of the *L. donovani* DNA topoisomerase IB enzyme (LdTopIB) and the potency of LdTopIB inhibition is chain length dependent. We also demonstrate the generality of the antiprotozoal activity of 2-HDA and 2-ODA against *P. falciparum*, and review our present knowledge of their inhibition of key *P. falciparum* enzymes such as PfFabZ, PfFabG, and PfFabI together with some possible modes of inhibition. Recent research by our group has also demonstrated that 2-ODA displays antineoplastic activity, specifically against the neuroblastoma SH-SY5Y cell line via lactate dehydrogenase (LDH) release, which is a cell death mechanism principally associated to necrosis. This is the first comprehensive review of the medicinal chemistry of this interesting group of acetylenic fatty acids.

Keywords

2-Alkynoic fatty acids; Leishmaniasis; *Leishmania donovani*; Malaria; Necrosis; Neuroblastoma; *Plasmodium falciparum*; Topoisomerase IB

1. Introduction

The acetylenic fatty acids are an interesting group of natural products as well as of synthetic compounds that have attracted the attention of lipid chemists, natural products chemists as well as of medicinal chemists for the variety of biological activities that they display. Excellent reviews have appeared on the chemistry and medicinal potential of this interesting group of fatty acids, but more recently the review on the subject by Minto and Blacklock is noteworthy mentioning (Minto and Blacklock, 2008).

Many of the known acetylenic fatty acids are natural products (terrestrial and marine) and some believe that the number of these natural compounds can be well over six hundred. To name just a recent example, Xing-Cong Li et al. isolated a series of C₁₆-C₁₉ ⁶ acetylenic fatty acids from the plant *Sommera sabiceoides* and these fatty acids displayed potent in vitro antifungal activity against the dermatophytes *Trichophyton mentagrophytes* and *Trichophyton rubrum* as well as against the pathogens *Candida albicans* and *Aspergillus fumigatus* with minimum inhibitory concentrations (MIC) comparable to that of several control drugs (Li et al., 2008). Among these acetylenic fatty acids, the 6-nonadecynoic acid was identified as the most potent antifungal fatty acid in the series. Recent work has established that this fatty acid mediates its antifungal activity by disruption of fatty acid homeostasis (Xu et al., 2012).

Interesting is the biogenesis of the acetylenic bond in nature, which has elicited quite a number of interesting biosynthetic investigations. There are two acceptable biosynthetic pathways, which we can divide between a desaturation pathway and an elimination pathway (Minto and Blacklock, 2008). In the desaturation pathway an acetylenase can act directly on a double bond to introduce the third unsaturation involving NADPH and iron, such as is the case of a ⁶-acetylenase, among others. On the other hand, the elimination pathway just involves a de novo fatty acid synthesis by the elimination of pyrophosphate and CO₂ from a vinylic fatty acid (Minto and Blacklock, 2008).

2. The 2-alkynoic fatty acids

The aim of this review is to survey our past and present knowledge of the medicinal chemistry of the 2-alkynoic fatty acids, a rather simple class of acetylenic fatty acids with just one triple bond between the α and β carbons of the alkyl chain. None of these 2-alkynoic fatty acids are natural occurring compounds, but rather synthetic constructs by insightful lipid chemists. The 2-alkynoic fatty acids can be easily prepared from the deprotonation of a terminal alkyne with *n*-Buli in THF at -70°C followed by quenching of the anion with CO₂ and final protonation of the carboxylate with NH₄Cl (Scheme 1). The series of reported synthesized compounds to date ranges between a twelve-carbon fatty acid such as the 2-dodecynoic acid up to a twenty two carbon fatty acid such as the 2-docosynoic acid (Table 1). Despite their non-natural origin a great interest has aroused in the literature due to the interesting biological activities that they display. For example, Morbidoni et al. reported the antimycobacterial activity of a complete series of 2-alkynoic fatty acids against *Mycobacterium smegmatis* and a correlation was observed between the fatty acid antimycobacterial activity and the carbon chain length where the 2-octadecynoic acid (2-

ODA) displayed the best activity against *M. smegmatis* with a MIC of 4 μM (Morbidoni et al., 2006). Acids such as the 2-hexadecynoic acid (2-HDA), 2-octadecynoic acid (2-ODA), and the 2-nonadecynoic acid were also among the most toxic to *Mycobacterium tuberculosis* H₃₇Rv with MICs of 20-25 μM (Morbidoni et al., 2006). As to why these acids are toxic to mycobacteria the investigators found that 2-HDA and 2-ODA were able to inhibit InhA, the enoyl-ACP reductase from *M. tuberculosis*. In what turned out to be an exquisite mechanistic investigation it was also concluded that 2-HDA was metabolized into the 3-ketohexadecanoic acid, which inhibits fatty acid biosynthesis as well as into the 3-hexadecynoic acid, which inhibits β -oxidation (Fig. 1). Therefore, it was concluded that metabolites arising from 2-HDA were actually responsible for the observed antimycobacterial activities.

The fact that the 2-alkynoic fatty acids are inhibitors of lipid biosynthesis was recognized early in the literature (Robinson et al., 1963; Wood et al., 1980; Wood and Lee, 1981). The shorter-chain 2-alkynoic acids are potent irreversible inhibitors of the fatty acid synthase (FAS) and earlier studies on 2-HDA indicated that it inhibits the elongation of stearic and longer-chain fatty acids in rat liver microsomes (Wood and Lee, 1981). This work by Wood and Lee showed, in eukaryotes, that 2-HDA first gets activated by CoA into the corresponding CoA ester and then an isomerase converts the triple bond into the corresponding 2,3-allene (Fig. 2). The allene thus generated is an inhibitor of the enoyl CoA reductase responsible for the reduction of the (E)-2-hexadecenoic CoA ester, generated in the metabolism of 2-HDA, thus resulting in its accumulation (Wood and Lee, 1981). In a similar work by the same research group it was also found that 2-HDA inhibits the growth of cultured hepatoma 7288 (HTC) cells with a LD₅₀ of 35-85 μM . It was concluded that the observed reduced growth did not result from damaged plasma membranes, but rather that fatty acid elongation and acylation, especially triglyceride synthesis, was inhibited (Upreti et al., 1981).

The lipid biosynthesis inhibition displayed by the 2-alkynoic fatty acids does not seem to be limited to the aforementioned systems. In a rather interesting communication de Renobales et al. described the inhibition of the hydrocarbon biosynthesis in housefly microsomes by the 2-octadecynoate by interacting with the FAS system (de Renobales et al., 1986). In particular, the 2-octadecynoate inhibited the in vivo incorporation of [1-¹⁴C]acetate into both fatty acids and hydrocarbons. The data obtained by these researchers indicated that the elongation of 18:1 to longer chain fatty acids was also inhibited.

Earlier studies have also established that 2-HDA inhibits both the growth of bacterial and mammalian cells. For example, work by Konthikamee et al. demonstrated that 2-HDA was effective against Gram-positive cocci, such as *Staphylococcus aureus* (MIC = 21.4 μM), Gram-negative cocci, such as *Neisseria flava* (MIC = 21.4 μM), and Gram-negative rods, such as *Bacteroides fragilis* (MIC = 21.4 μM). However, 2-HDA was not effective against *Escherichia coli* or *Pseudomonas aeruginosa* (Konthikamee et al., 1982). In this work it was also shown that 2-HDA inhibited the growth of cultured HeLa cells. However, when palmitic acid was added to the medium, in conjunction with 2-HDA, the cellular inhibition was not observed (Konthikamee et al., 1982). No mechanistic work was described in this investigation.

The 2-alkynoic fatty acids can also display antifungal activity against Trichophyton mentagrophytes, Aspergillus niger, Trichoderma viridae and Myrothecium verrucaria (Gershon and Shanks, 1978). The fungitoxicity depends on the pH of the medium and at a given pH the chain length and pK_a of the acids are inversely related to the fungitoxicity. The authors found that the order of fungitoxicity, on a weight basis, follows the order: 2-alkynoic acids > 2-alkenoic acids > alkanolic acids > 2-bromoalkanoic acids > 2-fluoroalkanoic acids. The pH of the medium affects the fungitoxicity since at lower pHs the acids are less ionized and supposedly pass through the cell membrane more readily resulting in greater toxicity. To display its optimal fungitoxicity the 2-alkynoic fatty acid should have a chain length between 8 and 16 carbon atoms, while for a saturated fatty acid the optimal carbon chain length was determined between 4 and 10 carbon atoms. The authors indicate that what is important for fungitoxicity is the partition coefficient between lipid and water for membrane penetration rather than chain length or the pH of the medium. When the acidity of the acid is greater, then a larger lipophilic section or more methylene groups are needed for the acid to restore its ability to partition between lipid and water for effective membrane penetration. All things being equal a 2-alkynoic fatty acid has a pK_a of about 1.8 as compared to an alkanolic acid with a pK_a of 4.8 (Gershon and Shanks, 1978b). The authors conclude that the α and β carbon bond length is what influences the fungitoxicity rather than the pK_a (Gershon and Shanks, 1978b).

All things taken together it is clear that 2-HDA and 2-ODA are good antibacterial, antifungal, and antimycobacterial agents, but can also display cytotoxicity to cancer cells. Enzyme inhibition seems to be the preferred mechanism of action of these compounds whereby fatty acid biosynthesis is largely impacted. Given these results our research team was interested in studying the antiprotozoal activity of the 2-alkynoic fatty acids, in particular against Plasmodium falciparum, the key causative agent of malaria, and against Leishmania donovani, the causative agent of leishmaniasis. There were no studies in the literature describing the toxicity of the 2-alkynoic fatty acids against these parasites. Therefore, a review of what we have learned so far follows.

3. The antimalarial activity of the 2-alkynoic fatty acids

Malaria is largely a tropical disease. It is estimated by the World Health Organization (WHO) that around 655,000 deaths occur each year due to this disease (World Malaria Report 2012). Some of the most affected regions of the world include Africa, South East-Asia, and the eastern Mediterranean. Malaria is caused by a Plasmodium parasite, but of the four species of Plasmodium that can infect humans, Plasmodium falciparum is responsible for the most deadly form of the disease. There are three main therapeutic venues presently being developed to control malaria that include vaccine development, the use of artemisinin-based combination therapy (ACT), and pathogenesis (Douglas et al. 2010). We should mention that there are two different stages of the Plasmodium parasite that are important for therapeutic intervention, i.e., the blood stage and the liver stage. However, the toxicity of drugs presently being used to treat malaria, combined with the constant occurrence of resistant Plasmodium strains, has motivated the development of new combination drugs to tackle this disease.

Do fatty acids display antimalarial activity? In general, polyunsaturated fatty acids are the ones that display the best antimalarial activity. For example, Kumaratilake et al. observed that a high degree of unsaturation was critical for the antimalarial activity of fatty acids inasmuch as in killing *P. falciparum* at 20–40 $\mu\text{g/mL}$ (> 90% death) the order 22:6 (n-3) > 20:5 (n-3) > 20:4 (n-6) > 18:1 (n-9) > 22:0 was followed (Kumaratilake et al., 1992; Kumaratilake et al., 1997). These investigators concluded that the oxidized forms of the fatty acids were critical for the toxicity since when antioxidants were introduced in the medium the fatty acid-induced killing was reduced. Kumaratilake et al. also reported that negligible parasite killing was observed with oleic acid. However, in contrast to what was reported in the latter work Krugliak et al. observed that in killing the FCR3 strain of *P. falciparum* a series of C_{18} fatty acids followed the order: oleic acid ($\text{IC}_{50} = 23 \mu\text{g/mL}$) > linoleic acid ($\text{IC}_{50} = 76 \mu\text{g/mL}$) > linolenic acid ($\text{IC}_{50} = 92 \mu\text{g/mL}$), which at first glance seems to be in contradiction to the observations previously reported by Kumaratilake et al. (Krugliak et al., 1995). Nevertheless, in the Krugliak work a strong effort was made in trying to understand the mechanism behind the observed antimalarial effect displayed by the C_{18} fatty acids, but no clear mechanism of action could be determined. However, it was clear that the toxicity displayed by the fatty acids was rather quick and full inhibition of nucleic acids and protein syntheses was observed in less than 30 min, but no hemolysis of infected cells, no effect on lipid peroxidation and ATP levels was observed.

Some simple naturally occurring acetylenic fatty acids also display antimalarial activity. For example, the fatty acid scleropyric acid, a C_{18} fatty acid with a triple bond at C-13 and a double bond at C-17 was isolated from the twigs of *Scleropyrum wallichianum*, and displays toxicity towards a K1 multidrug-resistant *P. falciparum* with an $\text{IC}_{50} = 7.2 \mu\text{g/mL}$ (Suksamrarn et al., 2005). These results indicate that acetylenic fatty acids could be worth studying as promising toxic substances against the *P. falciparum* parasite. Given the multitude of cytotoxic activities displayed by the 2-alkynoic fatty acids, in particular those displayed by 2-HDA and 2-ODA, a logical next step was to test these compounds against the *P. falciparum* parasite and look for possible mechanisms of action.

In collaboration with Dr. Deniz Tasdemir of the School of Chemistry of the National University of Ireland, Galway, and other research groups, our team tested a series of C_{16} isomeric acetylenic fatty acids against a *P. falciparum* K1 resistant strain and against *Plasmodium yoelii*, and found that 2-HDA was the most toxic and effective fatty acid ($\text{IC}_{50} = 41.2 \mu\text{M}$) against these plasmodium parasites (Tasdemir et al., 2010). Immunofluorescence microscopy studies of *P. yoelii*-infected HepG2:CD81 cells showed that 2-HDA was the only C_{16} acetylenic fatty acid interacting with the liver stage parasites detected by using an antibody against the HSP70 protein (Tasdemir et al., 2010). Moreover, it was also observed by detailed fluorescence microscopy images of *P. yoelii* liver stage schizonts that as the concentration of 2-HDA was increased, the parasite was systematically reduced. Recent studies by our team also indicate that there is a chain length dependence on the effectiveness of the 2-alkynoic fatty acids against *Plasmodium* parasites. For example, 2-ODA is toxic to *P. falciparum* at an $\text{IC}_{50} = 20 \mu\text{M}$ followed by 2-HDA ($\text{IC}_{50} = 41.2 \mu\text{M}$) and finally by the 2-tetradecynoic acid (2-TDA) with an $\text{IC}_{50} = 121.7 \mu\text{M}$ (Carballeira and Tasdemir, unpublished data).

Given the fact that 2-HDA was able to intervene with liver stage Plasmodium parasites, it then became logical to explore the potential of these compounds to inhibit key enzymes responsible for the fatty acid biosynthetic machinery within the parasite. Interesting is the fact that Plasmodium utilizes the so-called type II fatty acid synthase (FASII) system for fatty acid biosynthesis. In the FASII system there are many key enzymes, at least eight, that participate in the biosynthesis of fatty acids such as enoyl reduction (FabI), β -ketoacyl reduction (FabG), and β -hydroxyacyl dehydration (FabZ) to just name a few (Fig. 3). This is in contrast to what normally takes place in mammalian fatty acid synthesis where a single fatty acid synthase with six catalytic sites can take care of 42 catalytic steps and build up to 18-carbon fatty acid chains (Maier et al. 2008). This difference makes it quite feasible to inhibit the plasmodium Fabs (PfFab) without damaging the host mammalian fatty acid synthetic machinery. If fatty acid biosynthesis is inhibited within the parasite, then it will starve to death. Therefore, it became of interest to us to test the 2-alkynoic fatty acids 2-TDA, 2-HDA, and 2-ODA as possible inhibitors of the plasmodium enzymes PfFabI, PfFabZ, and PfFabG involved in type II fatty acid biosynthesis (Table 2). As can be seen from Table 2 both 2-HDA and 2-ODA are good inhibitors (IC_{50} s 1–2 μ M) of PfFabI and PfFabZ (Tasdemir et al., 2010; Carballeira and Tasdemir, unpublished data). As a comparison, this degree of inhibition cannot be obtained with a saturated fatty acid such as palmitic acid.

Why are the 2-alkynoic fatty acids relatively good inhibitors of the PfFab enzymes and why there is chain length dependence? There are several plausible mechanisms to be considered that could give some answers. One possible mechanism (Fig. 4) is inactivation of the enzyme by a nucleophilic amino acid present in the enzyme (such as histidine) which could attack either the 2-alkynoic fatty acid directly in a Michael fashion or less likely attack a 2,3-allene known to be generated in situ by other 2-alkynoic or 3-alkynoic substrates (Fendrich and Abeles, 1982; Kostrewa et al. 2005; Wu et al., 2008). This covalent linkage could lead to enzyme inactivation. However, kinetic studies carried out by the Tasdemir group indicated that 2-HDA inhibits PfFabI in a non-competitive manner with respect to crotonyl-CoA and the cofactor NADH (Tasdemir et al., 2010). Therefore, 2-HDA must be binding to PfFabI at a site distinct of the substrate or the cofactor site (Tasdemir et al., 2010). On the other hand, kinetic studies also revealed that 2-HDA, with respect to the substrate, competitively inhibit PfFabZ, and this enzyme does not require a cofactor. Therefore, it seems that the 2-alkynoic fatty acids are just hydrogen bonding in these enzymes to a region separate from the active site and in doing so alter the conformation of the enzyme in such a way that it inhibits its function or the acids are simply blocking the active site. However, these enzymatic studies do not necessarily prove that this is the mechanism by which these compounds kill the parasites since the inhibition displayed by the acetylenic fatty acids on the purified enzymes does not necessarily translates into the same inhibition when dealing with the same enzymes “in vivo”.

The group of Dr. Adriano D. Andricopulo and Dr. Rafael V. C. Guido of the University of São Paulo in Brazil recently performed molecular modeling of both 2-ODA and 2-TDA docked into the active site of PfFabZ (Fig. 5). The model clearly revealed that both fatty acids are able to adopt extended conformations into the PfFabZ active site and non-polar interactions play a key role in ordering the inhibitors within the binding cavity. The fatty

acid alkyl chains can make favorable Van der Waals interactions with several amino acids within the active site. The difference in reactivity between 2-ODA and 2-TDA could be due to the fact that the carboxyl groups of the two fatty acids seem to bind to different regions within the active binding site. In the case of 2-TDA the critical hydrogen bonding occurs to the NHs of a Gly142 and a Val143, while 2-ODA interacts more favorably with the NH₂ of a Gln145 and to two NHs of a Lys181 and a Val183. In addition, the triple bond in 2-ODA orients the carboxyl end of the acetylenic fatty acid into a hydrophobic cavity favoring additional hydrophobic interactions (Carballeira and Andricopulo, unpublished data). These results could explain why 2-ODA and 2-HDA are good inhibitors of PfFabZ, while 2-TDA and other fatty acids are not. However, these results are valid if the 2-alkynoic fatty acids do not get metabolized into other compounds in *Plasmodium*. Therefore, further work is warranted as to the metabolism of these compounds in *P. falciparum*.

4. The antileishmanial activity of the 2-alkynoic fatty acids

Visceral leishmaniasis is mainly a tropical disease caused by *L. donovani*, a parasite transmitted by the sand fly (McCall et al., 2013). This parasite can invade the spleen and liver macrophages causing anemia, fever and ultimately death if left untreated. There are two stages of *L. donovani*, the promastigotes, which are the ones that are initially injected into the bloodstream by the sand fly, and the amastigotes, which are the result of the transformation of promastigotes inside macrophages. There are approximately 500,000 cases of leishmaniasis each year distributed in countries such as India, Bangladesh, Indonesia, and the Sudan. The disease has also been an important problem for US troops overseas in areas such as the Middle East, Africa, and Afghanistan. At present there are several therapeutic drugs available to fight the parasite such as meglumine antimonite, a pentavalent antimonial, and miltefosine, an oral chemotherapeutic drug. However, as we have seen with the plasmodium parasite, the emergence of resistant *L. donovani* strains coupled to the toxicity of available drugs outlines the need to look for newer alternatives to fight leishmaniasis. The key is a drug that could kill the parasite without harming the mammalian host.

The antileishmanial activity of fatty acids has been recognized for many years. Two publications are representative of this effort. Cunningham et al. reported that n-decanoic acid, n-dodecanoic acid, and n-hexadecanoic acid, at levels of 100 µg/mL, inhibited the motility of *L. donovani* promastigotes but oleic acid was reported to have no effect on the parasite (Cunningham et al., 1972). Chaudhuri et al. investigated a series of C₄-C₁₈ fatty acids against *L. donovani* promastigotes and found that the C₁₈ fatty acids were the most inhibitory and the unsaturated fatty acids displayed more toxicity than their corresponding saturated analogs. For example, oleic acid displayed a MIC of 0.09 µmol/ml while octadecanoic acid resulted in a MIC of 1.39 µmol/ml. Therefore, as we noted before in the case of *P. falciparum*, fatty acids should contain a high degree of unsaturation to display antileishmanial or antiprotozoal activity (Chaudhuri et al., 1986).

Our research team explored the antileishmanial activity of acetylenic fatty acids. The naturally occurring 6-heptadecynoic and 6-icosynoic acids displayed good antiprotozoal activity towards *L. donovani* promastigotes (EC₅₀ = 1-6 µg/ml) with the 6-icosynoic acid being the most effective in the series. The saturated fatty acids n-heptadecanoic acid and n-

eicosanoic acid were not effective towards *L. donovani* indicating that the unsaturation was necessary for the observed leishmanicidal activity. In addition, the 6-icosynoic acid was a good inhibitor of the leishmania DNA topoisomerase IB enzyme (LdTopIB) with EC_{50} s between 36–49 μ M. LdTopIB is a plausible intracellular target for these fatty acids and this was the first study assessing acetylenic fatty acids as inhibitors of LdTopIB (Carballeira et al., 2009). The trypanosomal and leishmanial type IB DNA topoisomerases differ significantly from the homologous mammalian structures since they are phylogenetically unique and possess an anomalous dimeric structure (Reguera et al. 2006). These enzymes have been recognized as excellent targets for the development of antiparasitic drugs. It is interesting to mention that the *P. falciparum* topoisomerase I (PfTopI) is a 104 kDa protein with 839 amino acids and it has a 42% homology to the human enzyme. Therefore, the LdTopIB is significantly different from the PfTopIB.

Based on the toxicity displayed by the acetylenic fatty acids it then became logical to test 2-HDA, 2-ODA, and 2-TDA, against *L. donovani* promastigotes. Interestingly, a similar cytotoxic effect as that found with the *P. falciparum* parasite was found here, i.e., 2-ODA was the most effective of the series ($IC_{50} = 11.0 \mu$ M) together with 2-HDA ($IC_{50} = 17.8 \mu$ M), but 2-TDA was the least effective of the series ($IC_{50} = 24.7 \mu$ M) (Carballeira et al., 2012). As a possible mechanism of toxicity apoptosis was tested in *Leishmania infantum* promastigotes where 2-HDA displayed an $IC_{50} = 14.9 \mu$ M by detecting the translocation of phosphatidylserine (PS) to the cell surface with the Annexin-FITC reagent (Carballeira et al., 2012). It is known that *Leishmania* amastigotes can fake its own death to gain access to macrophages by exposing PS on its surface (de Freitas Balanco et al. 2001). However, no significant concentration of apoptotic cells was observed for neither of the 2-alkynoic acids tested. Therefore, apoptosis is not a mechanism by which the 2-alkynoic acids kill these promastigotes.

Aimed at assessing a plausible mechanism of toxicity our team, in collaboration with Dr. Rafael Balaña-Fouce and Dr. Rosa Reguera from the Department of Biomedical Sciences of the University of León, Spain, studied the inhibition of the relaxation activities of LdTopIB and hTopIB by the 2-alkynoic fatty acids (Table 3). Two interesting findings can be clearly elucidated from the data. Among the series of 2-alkynoic fatty acids tested, the 2-ODA was the most inhibitory of the series ($EC_{50} = 5.3 \mu$ M) followed by 2-HDA and finally 2-TDA. The second interesting finding is that LdTopIB is more sensitive to the inhibition by the 2-alkynoic fatty acids than hTopIB. This seems to indicate that it will be possible to preferentially intervene with the leishmanial enzyme over the human enzyme (Carballeira et al., 2012). It should be mentioned that none of the tested 2-alkynoic fatty acids were toxic to BALB/c murine macrophages (Table 3).

Although there seems to be a correlation between the toxicity of the C_{14} – C_{18} 2-alkynoic fatty acids towards *L. donovani* and their inhibition of LdTopIB, this apparent correlation does not necessarily prove that topoisomerase I inhibition is the preferred death mechanism. However, the question still holds as to what is the LdTopIB inhibition mechanism displayed by the 2-alkynoic fatty acids? At this stage, with the available experimental data, we can only speculate. It is very likely that the 2-alkynoic fatty acids follow a similar inhibitory mechanism as that proposed by Castelli et al. for the interaction of cEPA with hTopIB

(Castelli et al., 2009). The fatty acid is probably not binding to the DNA-topoisomerase binary complex, but it may be interacting directly with the TopIB enzyme. The 2-alkynoic fatty acids could be just binding in close proximity to the active site thus inhibiting DNA cleavage by simply blocking the nucleophilic attack of the tyrosine on the DNA phosphate (Castelli et al., 2009). More mechanistic studies are indeed needed to fully elucidate the mechanism by which these 2-alkynoic fatty acids inhibit topoisomerases and why there is a chain length preference.

5. The anticancer potential of the 2-alkynoic fatty acids

The anticancer activity of natural and synthetic acetylenic lipids is well recognized (Dembitsky, 2006). Previously we mentioned that 2-HDA inhibits the growth of HeLa cells as well as a cultured hepatoma 7288 (HTC) cell line (Konthikamee et al., 1982; Upreti et al., 1981). Moreover, we have shown that 2-ODA is able to inhibit hTopIB with an EC_{50} of 34.8 μ M, while 2-HDA needs higher concentrations to inhibit hTopIB (Carballeira et al., 2012). In fact, we determined for 2-HDA an IC_{50} of 361.0 ± 1.0 μ M as the best value to cause the inhibition of a human topoisomerase I (Carballeira and Sanabria, unpublished data). Therefore, the potential of 2-ODA as an anticancer compound is worth investigating.

Our team recently chose to investigate 2-ODA against the human SH-SY5Y neuroblastoma cell line. The human SH-SY5Y neuroblastoma cell line was originally established from the bone marrow biopsy of a four year-old female neuroblastoma patient (Biedler et al., 1973). It is a well-established in vitro cell model for neuronal differentiation, a process in which a less specialized cell (immature cell) develops into a specialized cell type (mature cell) in response to a specific trigger. All-trans retinoic acid (RA) induces neuronal differentiation in neuroblastoma cells, a process called neuritogenesis. We found that 2-ODA is cytotoxic to the human SH-SY5Y neuroblastoma cell line at an IC_{50} of 26 ± 2 μ M (Carballeira and Orellano, unpublished data). Basically, the SH-SY5Y cells were differentiated with 15 μ M RA for 5 d and then 2-ODA was added to the cultured media. The activity of the acid was assessed by means of a sulforhodamine B (SRB) assay. On the other hand 2-ODA was not as effective against a mouse leukaemic monocyte macrophage cell line (RAW 264.7) since it displays an IC_{50} of 45.5 ± 3.4 μ M and against a human colon adenocarcinoma grade II cell line (HT29) with an IC_{50} of 101.1 ± 0.9 μ M (Carballeira and Balaña-Fouce, unpublished data). Therefore, we chose the SH-SY5Y neuroblastoma cell line to study the mechanisms of cytotoxicity induced by 2-ODA besides hTopIB inhibition.

Our team explored the mechanism of cytotoxicity of 2-ODA towards neuroblastoma cells by probing two basic death mechanisms, i.e., apoptosis and necrosis (Carballeira and Orellano, unpublished data). These were the first studies on the mechanism of toxicity of these fatty acids on neuroblastoma cells since a precise mechanism of action is not available. Apoptosis has been recognized as the major mechanism of cell death in the nervous system (Deshmukh and Johnson, 1997) and the role of apoptosis as a result of exposure to chemotherapeutic agents and pathological conditions is well documented. The main hallmark of apoptosis is the orchestrated activation of a biochemical cascade, which activates proteases that destroy molecules required for cell survival, and other molecules that mediate a program of cell suicide (Friedlander, 2003). These critical players are aspartate-specific cysteine proteases,

known as caspases (cysteiny, aspartate-specific proteases). In this regard, it is well documented that activating cleavage of caspase-3 is an important biochemical hallmark of apoptotic cell death. Following this reasoning, we used a caspase-3 screening (colorimetric CaspACE Assay System) to determine if apoptosis was one of the predominant cell death mechanisms occurring in our in vitro system. We found that 2-ODA did not produce any significant caspase-3 activation (Fig. 6, left).

Necrosis is a passive and unscheduled cell death mechanism resulting from environmental perturbation with uncontrolled release of inflammatory cellular contents (Fink and Cookson, 2005). This cell death mechanism is triggered by detrimental stimuli such as toxins, severe hypoxia, massive insult, and conditions resulting in ATP depletion (Hetts, 1998). Mitochondrial and nuclear swelling rupture of the plasma membrane, condensation of chromatin around the nucleus, and random DNA cleavage characterize necrotic cell death. One of the key features of necrosis, which could also happen with apoptosis, is increased plasma membrane permeability resulting in the loss of key cell components such as the enzyme lactate dehydrogenase (LDH), which is released into the culture medium following loss of membrane integrity. We measured the leakage of LDH in neuroblastoma cells after being treated with 2-ODA using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. This LDH assay is the coupling of two reactions. In the first reaction the released LDH catalyzes the oxidation of lactate to pyruvate by the reduction of NAD^+ to NADH. In the second step diaphorase uses the NADH as catalyst for the conversion of a tetrazolium salt to formazan, whose formation can be followed by absorption at 490-520 nm. In this study, 2-ODA was able to elicit significant LDH release (Fig. 6, right). Therefore, necrosis seems to be the preferred mechanism by which 2-ODA elicits the cell death of the neuroblastoma cells.

6. Conclusions

From the information obtained so far it is clear that the 2-alkynoic fatty acids, in particular 2-HDA and 2-ODA, display antiprotozoal activity against *P. falciparum*. There is a fatty acyl chain length dependence with 2-ODA being the best candidate discovered so far among those tested in the series. As exemplified by the work with 2-HDA on *P. yoelii*, the 2-alkynoic fatty acids are also effective against liver stage plasmodium infections. Therefore, it was reasonable to study their inhibition against the PfFab enzymes. 2-ODA and 2-HDA displayed the strongest inhibition of PfFabI, PfFabZ, and PfFabG indirectly supporting earlier studies that the 2-alkynoic fatty acids can inhibit fatty acid biosynthesis in microorganisms and protozoa. Molecular modeling studies are providing valuable insights that help explain the different affinities displayed by the 2-alkynoic acids towards the PfFab enzymes.

The present findings reveal that the 2-alkynoic fatty acids are more toxic to *L. donovani* than towards *P. falciparum*. A possible intracellular target for these compounds is the topoisomerase IB enzymes, in particular LdTopIB. We have shown that the 2-alkynoic fatty acids are good inhibitors of LdTopIB and that there is also an alkyl chain dependence being 2-ODA the best inhibitor to date. Based on all of the fatty acids tested so far, it is clear that LdTopIB is more sensitive towards inhibition by the 2-alkynoic fatty acids than hTopIB. Therefore, it should be possible to interfere with LdTopIB without damaging hTopIB.

It was shown that 2-ODA is the best inhibitor of hTopIB among the 2-alkynoic fatty acids tested to date. This immediately proposes a possible anticancer potential for 2-ODA. It was demonstrated that 2-ODA displays cytotoxicity towards different cancer cell lines, but it was particularly effective towards the neuroblastoma SH-SY5Y cell line. Necrosis seems to be the preferred death mechanism induced by 2-ODA in neuroblastomas. There is no doubt that future work should give us more insight as to the potential of the 2-alkynoic fatty acids as antiprotozoal and anticancer agents. In particular, it will be worthwhile to study the biosynthesis of fatty acids in these organisms in the presence of the 2-alkynoic fatty acids. In addition, it will be important to assess the metabolism of the 2-alkynoic fatty acids in the parasites responsible for malaria and leishmaniasis.

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Highlights

- This is first comprehensive review of the medicinal chemistry of the 2-alkynoic fatty acids.
- These compounds display antimycobacterial, antifungal, anticancer, and antiprotozoal activities.
- This review attempts to collect all of the available information in the literature so that a clearer understanding of the potential of these fatty acids could be envisaged and at the same time open the door for future works in the field.

Scheme 1.

Synthesis of the 2-alkynoic fatty acids with representative yields. Taken from Carballeira et al. 2012.

Fig. 1.
Proposed metabolism of 2-hexadecynoic acid into 3-ketohexadecanoic acid and 3-hexadecynoic acid by *M. tuberculosis*. Adapted from Morbidoni et al., 2006.

Fig. 2.

Proposed inhibition of fatty acid elongation in rat liver microsomes by 2-HDA. Adapted from Wood and Lee, 1981.

Fig. 3.

A typical type II fatty acid synthase (FASII) system and the key enzymes involved in the process.

Fig. 4. Postulated enzyme inactivation mechanisms promoted by nucleophilic attacks on 2-alkynoic CoA esters resulting in covalent bond formation. Taken from Fendrich et al., 1982 and Wu et al., 2008.

Fig. 5.

Predicted binding mode of 2-ODA (dark gray) and 2-TDA (light gray) into the binding site of PfFabZ. Left: inhibitors and protein residues involved in the ligand-receptor binding are indicated as ball-and-stick and stick models, respectively. Chain A and Chain B residues are indicated as green and cyan, respectively, and hydrogen bonds are illustrated as yellow dashed lines. Right: view of the active site showing the solvent accessible surface of PfFabZ and the spatial complementarity of the inhibitors. Pictures and text courtesy of Dr. Adriano D. Andricopulo and Dr. Rafael V.C. Guido of the University of São Paulo, Brazil (unpublished data).

Fig. 6.

(left). Caspase-3 activation in treated human SH-SY5Y neuroblastoma cells. Cultures were treated with 500 μ M hexadecanoic acid (16:0) and 40 μ M 2-octadecynoic acid (18:1- Δ^2) for 48h. Cultures were also treated with 15 μ M etoposide (E) and etoposide plus 50 μ M pan-caspase inhibitor Z-VAD-FMK (I) served as positive and negative controls. Data are the means $\pm$ SEM of values from at least three independent experiments. Treated cells were compared to non-treated cells, control (one-way ANOVA). Fig. 6. (right). LDH release in treated human SH-SY5Y neuroblastoma cells. Cultures were treated with 500 μ M hexadecanoic acid (16:0) and 40 μ M 2-octadecynoic acid (18:1- Δ^2) for 48h. Cultures treated with 15 μ M etoposide (E) served as a positive control. Data are the means $\pm$ SEM of values from at least three independent experiments. Treated cells were compared to non-treated cells, control (one-way ANOVA). (Carballeira and Orellano, unpublished data).

Table 1Antimycobacterial activity of the 2-alkynoic fatty acids against *Mycobacterium smegmatis*.

Compound	<i>M. smegmatis</i> mc ² 155 (μM) ^a
2-Dodecynoic acid	>300
2-Tetradecynoic acid	266
2-Hexadecynoic acid	10
3-Hexadecynoic acid	20
4-Hexadecynoic acid	30
5-Hexadecynoic acid	80
2-Octadecynoic acid	4
3-Octadecynoic acid	36
4-Octadecynoic acid	>300
5-Octadecynoic acid	360
2-Nonadecynoic acid	35
2-Eicosynoic acid	>300
2-Docosynoic acid	>300
Isoniazid	40
2-trans-Octadecenoic acid	>200
9-cis-Octadecenoic acid	>200

^aData adapted from Morbidoni et al., 2006.

Table 2

Inhibitory activity of the 2-alkynoic fatty acids against PfFabs (IC₅₀ values in μM)^a.

Fatty acids	<i>P. falciparum</i> K1	PfFabI	PfFabI	PfFabG
2-TDA	121.7	5.3	15.6	24.5
2-HDA	41.2	1.5	2.3	13.9
2-ODA	21.7	1.1	1.0	2.9
16:0	>100	>100	>100	>100

^a Carballeira and Tasdemir, unpublished data.

Table 3

Inhibition of the relaxation activities of LdTopIB and hTopIB by the 2-alkynoic fatty acids and their toxicity towards murine macrophages (values in μM)^a.

Fatty acids	LdTopIB	hTopIB	Murine macrophages
	EC ₅₀	EC ₅₀	BALB/c IC ₅₀
2-TDA	67.8 $\pm$ 1.1	> 100	> 100
2-HDA	28.7 $\pm$ 1.3	> 100	> 100
2-ODA	5.3 $\pm$ 0.7	34.8 $\pm$ 2.3	> 100
CPT	0.67 $\pm$ 0.08	2.0 $\pm$ 1.0	0.62 $\pm$ 0.13

^aData taken from Carballeira et al., 2012. CPT = camptothecin.