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COMMUNICATION

The (5*Z*)-5-Pentacosenoic and 5-Pentacosynoic Acids Inhibit the HIV-1 Reverse Transcriptase

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Abstract The natural fatty acids (5*Z*)-5-pentacosenoic and (9*Z*)-9-pentacosenoic acids were synthesized for the first time in eight steps starting from either 4-bromo-1-butanol or 8-bromo-1-butanol and in 20–58 % overall yields, while the novel fatty acids 5-pentacosynoic and 9-pentacosynoic acids were also synthesized in six steps and in 34–43 % overall yields. The Δ^5 acids displayed the best $IC_{50's}$ (24–38 μM) against the HIV-1 reverse transcriptase (RT) enzyme, comparable to nervonic acid ($IC_{50} = 12$ μM). The Δ^9 acids were not as effective towards HIV-RT with the (9*Z*)-9-pentacosenoic acid displaying an $IC_{50} = 54$ μM and the 9-pentacosynoic acid not inhibiting the enzyme at all. Fatty acid chain length and position of the unsaturation was important for the observed inhibition. None of the synthesized fatty acids were toxic ($IC_{50} > 500$ μM) towards peripheral blood mononuclear cells. Molecular modeling studies indicated the structural determinants underlying the biological activity of the most potent compounds. These results provide new insights into the structural requirements that must be present in fatty acids so as to enhance their inhibitory potential towards HIV-RT.

Keywords Fatty acids · Human immunodeficiency virus · Pentacosenoic acid · Reverse transcriptase · Synthesis

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
FA	Fatty acids
GC-MS	Gas chromatography-mass spectrometry
HAART	Highly active-antiretroviral therapy
HIV	Human immunodeficiency virus
IC_{50}	Inhibitory concentration for half-life maximal inhibition
NA	Nervonic acid
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
PBMC	Peripheral blood mononuclear cells
PDC	Pyridinium dichromate
p-TSA	*p*-Toluenesulfonic acid
RT	Reverse transcriptase
THF	Tetrahydrofuran

Introduction

The potential of fatty acids to combat infectious diseases such as malaria, tuberculosis, and fungal infections

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continues to be a focus of recent research activity [1]. Fatty acids are important in a diversity of pathological conditions and have been postulated as possible drug candidates against viruses, bacteria, fungi, and cancerous cells by inducing death responses [1–6]. Numerous fatty acids, mainly of bacterial origin, also inhibit DNA polymerase and topoisomerase I, and display antimalarial and antiviral activity [1, 7–11].

The World Health Organization (WHO) considers the human immunodeficiency virus (HIV) infection pandemic [12]. In 2012 alone, around 2.3 million adults and children were infected with HIV, bringing the total population living with HIV to 35.3 million, mostly on the African continent [12, 13]. Even after more than twenty years of research, a HIV vaccine has yet to be discovered and current research focuses on the search for novel anti-HIV agents [14]. There are more than twenty antiretroviral drugs approved for clinical use today [15], but due to their long-term use in drug therapies these drugs must be relatively nontoxic [16]. This implies that our present arsenal to combat the disease is limited due to adverse effects and toxicities that normally arise from long-term use coupled to the emergence of drug resistance [14]. Since HIV-1 can acquire drug resistance to any single inhibitor quite easily, multiple drugs are typically used simultaneously [14].

The reverse transcriptase (RT) is a multifunctional enzyme that plays a key role in HIV-1 replication [16–22]. It possesses distinct DNA polymerase and RNase H activities, which are used to convert the viral RNA genome into double-stranded linear proviral DNA that are subsequently integrated into the host genome [16–24]. Since RT inhibitors prevent the RNA-DNA conversion, a vital role of the viral life cycle, it is no wonder that more than half the currently approved HIV-1 antiretrovirals are RT inhibitors [21]. There are two classes of RT inhibitors, namely nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) [15, 25–27]. Together they are the backbone of the highly active-antiretroviral therapy (HAART) regime [24], the standard treatment that usually combines two NRTI with one NNRTI or with one protease inhibitor (PI) [14, 16, 24]. However, over the years, NNRTI have become more popular than the PI [21].

Even though the NNRTI are a chemically heterogeneous group of compounds, they possess a common mode of binding and they all bind to the same site on the RT enzyme [28, 29]. This binding site is located in the palm sub-domain between two beta-sheets of the p66 subunit about 10 Å away from the DNA polymerase catalytic site and approximately 60 Å from the RNase H active site of the p66 subunit [24, 28, 30].

The HAART regime currently used is not potent enough to completely suppress virus replication to the point of eradication due to the development of drug-resistance [13, 25]. It is still necessary to develop new less cytotoxic antiretrovirals for the treatment of this disease. Natural products from various marine sources have been known to possess diverse biological activities including antiviral activity [11, 31].

Nervonic acid (NA) is a *cis*-15-tetracosenoic acid found in the sphingolipids of white matter in human brain where it plays an important role in the biosynthesis of nerve cell myelin [32]. NA is also abundantly found in the spotted seal, king salmon and beluga whale. NA is used to treat adrenoleukodystrophy and multiple sclerosis where the decreased level of nervonic acid in sphingolipids causes demyelination [32]. This FA possesses great inhibitory activity against several enzymes involved in replication [33–36]. Mizushina and co-workers discovered that NA was a potent non-competitive inhibitor of HIV-1 RT with an IC_{50} of 4.8 μM and almost complete inhibition (more than 80 %) at 8 μM [36]. Modification of the carboxyl group of NA to a carboxyl ester results in the loss of inhibitory activity thus indicating that the carboxyl group in NA is important for RT inhibition [33]. Therefore, the carboxyl group and length of the alkyl chain in NA play crucial roles in HIV-RT inhibition.

Since nervonic acid is a potent HIV-1 RT inhibitor, other monounsaturated long chain fatty acids could be potential HIV-1 RT inhibitors as well. The pentacosenoic acids are a minor class of lipids found in a wide range of organisms including bacteria and marine sponges [37]. The (5*Z*)-5-pentacosenoic acid (**2a**) is naturally found in the marine sponge *Pseudaxinella cf. lunaecharta* and in the bacterium *Mycobacterium tuberculosis* [38, 39]. On the other hand, the (9*Z*)-9-pentacosenoic acid (**2b**), which is more ubiquitous, was identified in various sponges including the marine sponges *Dysidea fragilis, Desmapsamma anchorata, Geodinella robusta, Spheciospongia cuspidifera* and *Hymeniacidon sanguinea* as well as in the bacterium *Mycobacterium tuberculosis* [38, 40–44]. These long-chain monounsaturated fatty acids have the potential to be RT inhibitors and our aim was to explore them as such. However, since the isolation and purification of these lipids from natural sources, in reasonable quantities, is an extremely difficult task, they must be synthesized if we are to explore their full biological potential.

In this work, we synthesized, for the first time, the naturally occurring fatty acids **2a** and **2b** as well as the alkynoic analogs 5-pentacosynoic (**1a**), 9-pentacosynoic (**1b**), and 2-pentacosynoic acids. Their HIV-RT inhibitory activity was determined and compared to other shorter chain analogs. Molecular modeling studies indicated the structural basis underlying the inhibitory activities of the most potent compounds.

Materials and Methods

Synthesis of the Fatty Acids

The synthetic experimental procedures, the analytical and the spectral data of the products are presented in the supporting information.

Reverse Transcriptase Colorimetric Assay

The Reverse Transcriptase Colorimetric Assay Kit was purchased from the Roche Diagnostics Corporation, Indianapolis, Indiana. The assay was carried out according to the procedure described in Roche's Reverse Transcriptase Colorimetric Assay Kit manual with some minor modifications [45, 46]. First a 1000-μM stock solution of each of the fatty acids in dimethyl sulfoxide (DMSO) was prepared (final concentration of DMSO = 1 %). The stock solutions were sonicated for 1 h, and then each stock solution was further diluted to final concentrations of 100, 10, 1, 0.1 and 0.01 μM with Lysis buffer. The recombinant HIV-1-RT was briefly dissolved in Tris-buffer solution [50 mM Tris, 80 mM KCl, 2.5 mM DTT, 0.75 mM EDTA and 0.5 % Triton X-100, pH 7.8] to reach a final concentration of 2 ng/μL. The HIV-1-RT enzyme solutions with the experimental agents were incubated for 60 min at 37 °C. A 20 μL aliquot of a reaction mixture [incubation buffer: 50 mM Tris-buffer, containing 319 mM KCl, 33 mM magnesium chloride, and 11 mM DTT; nucleotides: 50 mM Tris–HCl at pH 7.8 with DIG-dUTP, biotin-dUTP and dTTP; and template: template/primer hybrid poly (A) × oligo $(dT)_{15}$] was added and incubated for 60 min at 37 °C. The reactions were transferred to microplate modules coated with streptavidin and incubated for 60 min at 37 °C. After the incubation the plate was washed 5 times with the kit's washing buffer followed by the addition of 200 μL of peroxidase-labeled antibody against digoxigenin (anti-DIG-POD) and incubated for 60 min at 37 °C. After that, the wells were washed five times with the kit's 250 μL washing buffer and incubated with 200 μL of ABTS substrate solution for about 10 min at rt (25 °C) at 250 rpm. The absorbance was taken at 405 nm (with reference wavelength at 490 nm) on a microplate reader (MRX II; Dynex Technologies, Chantilly, VA). The IC_{50} values were calculated using the Prism Software (Graphpad, San Diego, CA) from titration curves generated from the experimental values.

Molecular Modeling

The 3D structures of the inhibitors **1a** and **2a** were constructed using standard geometric parameters provided by the molecular modeling software package SYBYL 8.0. Single optimized conformation of each molecule was energetically minimized employing the Tripos force field [47] and the Powell conjugate gradient algorithm [48] with a convergence criterion of 0.05 kcal/mol/Å and Gasteiger–Hückel charges [49]. Molecular docking and scoring protocols were carried out with GOLD 5.1 (Cambridge Crystallographic Data Centre, Cambridge, UK) [50]. To investigate the binding mode of **1a** and **2a** GOLD 5.1 default parameters were used. The coordinates for HIV-1 RT solved at 2.8 Å (PDB ID 2HMI) [51] were used during the molecular modeling investigation. Hydrogen atoms were added in standard geometry using the GOLD 5.1 wizard. Histidine, glutamine, and asparagine residues within the binding site were manually checked for possible flipped orientation, protonation, and tautomeric states by the GOLD side chain wizard. The binding cavity of HIV-1 RT was defined as all the amino acid residues encompassed within a 20 Å radius sphere centered on the three dimensional coordinates (−4.029; 122.631; 11.880) of the side chain OH of Tyr188 in the p51 subunit. For each ligand the docking protocols were repeated twenty-five times. The ASP (Astex Statistical Potential) scoring function and visual inspection were employed to select the representative conformation for each inhibitor.

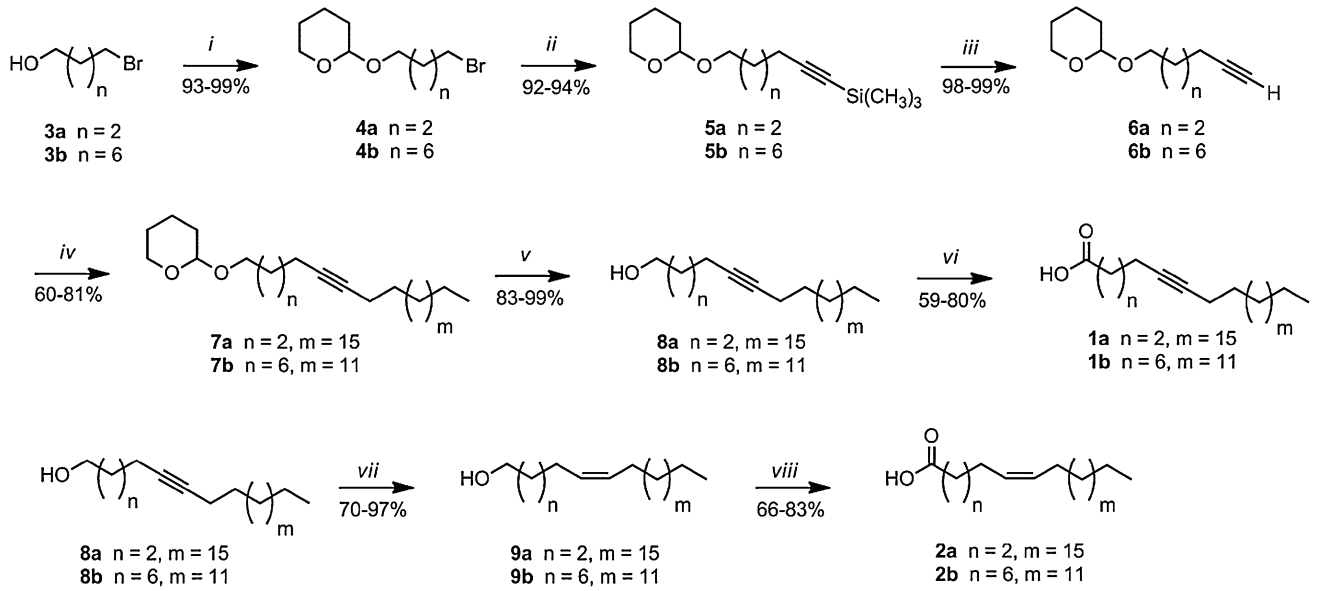
Cytotoxicity

The cytotoxicity of the unsaturated fatty acids against peripheral blood mononuclear cells (PBMC) was tested as described by Sanabria-Ríos *et al*. [52]. Briefly, PBMC were cultured in a culture medium supplemented with interleukin-2 (IL-2). These cells were seeded into a 96-well microplate (20,000 cells/200 μL/well) and fatty acids were added to the cell cultures. The final concentrations of fatty acids ranged from 5 to 500 μM. The cells were incubated at 37 °C for 3 days in a humidified 5 % CO_2 incubator. The cytotoxicity of the cells was evaluated by the MTT assay.

Results and Discussion

Synthesis

The 5- and 9-pentacosynoic acids **1a** and **1b** were prepared according to Scheme 1. The synthesis of **1a** and **1b** started with the protection of either commercially available 4-bromo-1-butanol (**3a**) or 8-bromo-1-octanol (**3b**) utilizing 3,4-dihydro-2*H*-pyran (DHP) in chloroform with catalytic amounts of *p*-toluenesulfonic acid (*p*-TSA) at rt for 3 h, affording **4a** or **4b** in 93–99 % yields. The alkyne coupling of **4a** or **4b** with (trimethylsilyl)acetylene using *n*-BuLi in THF-HMPA at −78 °C, afforded silanes **5a** or **5b** in 92–94 % yields. The removal of the trimethylsilyl



Scheme 1 Synthesis of the pentacosynoic acids **1a** and **1b**, and the pentacosenoic acids **2a** and **2b**. (*i*) DHP, *p*-TSA, $CHCl_3$, 3 h; (*ii*) *n*-BuLi, (trimethylsilyl)acetylene, HMPA, THF, −78 °C; (*iii*) TBAF, THF, 0 °C; (*iv*) 1-bromononadecane or 1-bromopentadecane, *n*-BuLi, HMPA, THF, −78 °C to 0 °C, 24 h; (*v*) *p*-TSA, MeOH, 45 °C, 3 h; (*vi*) PDC, DMF, rt, 24 h; (*vii*) H_2, Pd/C (10 %), quinoline, hexane; (*viii*) PDC, DMF, rt, 24 h

protecting group in **5a** or **5b** was achieved with tetrabutylammonium fluoride (TBAF) in THF, which resulted in the terminal alkynes **6a** or **6b** in 98–99 % yields. A second acetylenic coupling of **6a** with 1-bromononadecane or **6b** with 1-bromopentadecane using *n*-BuLi in THF-HMPA at −78 to 0 °C (the temperature was increased in order to increase the solubility of the bromo alkane which was added slowly for a period of 1 h) resulted in the alkynes **7a** or **7b** in 60–81 % yields. The deprotection of either **7a** or **7b** with *p*-TSA in methanol at 45 °C yielded the desired 5-pentacosyn-1-ol (**8a**) or 9-pentacosyn-1-ol (**8b**) in 83–99 % yields. Subsequent oxidation of **8a** or **8b** with pyridinium dichromate (PDC) in dimethylformamide (DMF) afforded the 5-pentacosynoic acid (**1a**) or the 9-pentacosynoic acid (**1b**) in 59–80 % yields. The overall yields for these six-step syntheses were 43 % for **1a** and 34 % for **1b**.

The natural products (5*Z*)-5-pentacosenoic acid (**2a**) and (9*Z*)-9-pentacosenoic acid (**2b**) were also synthesized as described in Scheme 1. This divergent synthetic procedure used the corresponding alkynols **8a** and **8b** obtained in the previous synthesis as starting points. Therefore, the synthesis of **2a** or **2b** started from the previously synthesized **8a** or **8b**, which were hydrogenated utilizing Lindlar's conditions (hydrogen gas, 10 % Pd/C and quinoline in hexane) in order to obtain the (5*Z*)-5-pentacosen-1-ol (**9a**) or the (9*Z*)-9-pentacosen-1-ol (**9b**) in 70–97 % yields. The pentacosenols **9a** or **9b** were oxidized with PDC in DMF at rt resulting in either **2a** in an 83 % yield or in **2b** in a 66 % yield. The synthesis of **2a** was completed with an overall yield of 58 % for the 8 steps (last two steps having a combined yield of 81 %), while the synthesis of **2b** was completed with an overall yield of 20 %. This is the first total synthesis for either **2a** or **2b**, which permits the full characterization of these fatty acids that were previously identified in nature by only gas chromatographic means [38–44].

HIV-RT Inhibitory Studies

As mentioned above our goal was to assess the natural fatty acids **2a** and **2b**, as well as their acetylenic analogs **1a** and **1b** as potential inhibitors of the DNA polymerase, reverse transcriptase (RT). In this regard, we modified an existing protocol for screening fatty acid candidates using a non-radioactive colorimetric assay method, which assesses the activity of HIV-1 RT [45, 46]. The colorimetric enzyme immunoassay quantitatively determines the retroviral reverse transcriptase activity by measuring the incorporation of digoxigenin- and biotin-labeled dUTP into DNA. The DNA molecule labeled with biotin nucleotides binds to the streptavidin coated MP module, followed by the binding of the peroxidase-labeled digoxigenin antibody molecule to the DNA molecule. Then, the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is added. The peroxidase catalyzes the cleavage of ABTS resulting in a colored product. The absorbance is directly correlated to

the amount of DNA synthesized, thus it is proportional to the level of RT activity.

In this study, RT inhibitory assays were performed in duplicate in a range of 0.01–1000 μM for each acid along with a positive control (no inhibitor of HIV-1 RT), negative control (no HIV-1-RT enzyme), inhibitor control (nervonic acid instead of experimental agent) and solvent control (DMSO instead of experimental agent). Our results first confirmed that DMSO can be used as solvent for the experimental agents at maximal and final concentration of 2 % with no effect on the activity of the HIV-1 RT. The results of the experimental agents were compared with a positive control, nervonic acid (NA), which according to our results displayed an IC_{50} of 12 ± 1 μM. The latter result supports previous studies indicating that NA inhibits HIV-1 RT in the micromolar range [36].

As starting point for our fatty acid structural studies, we used commercially available palmitic acid (16:0) as well as pentacosanoic acid (25:0). Our results revealed that the saturated (C_{16} or C_{25}) as well as other shorter chain Δ^6 acetylenic fatty acids (C_{17} or C_{20}) were the least potent of the acids tested (IC_{50} > 1000 μM). Among the acetylenic fatty acids evaluated (Table 1), acid **1a** was the best inhibitor of HIV-1 RT with an IC_{50} of 24 ± 1 μM. Surprisingly, in our hands, acid **1b** was not inhibitory towards the HIV-RT enzyme. When the position of the triple bond in the C_{25} acyl chain was changed from Δ5 to Δ2, a decrease in the inhibitory activity towards HIV-1 RT was also observed, as exemplified by an IC_{50} of 566 ± 1 μM displayed by the 2-pentacosynoic acid. Moreover, when a shorter chain (C_{17} or C_{20}) Δ^6 acetylenic fatty acid was tested, as exemplified by the natural fatty acids 6-heptadecynoic or 6-icosynoic acids [53], no inhibition of the HIV-1 RT enzyme was observed. Therefore, we can say that both fatty acid chain length and the triple bond position are directly related to the effectiveness of inhibition against the HIV-1 RT.

Table 1 The reverse transcriptase (RT) inhibitory activity of the tested fatty acids

Experimental agents	IC_{50} (μM)[a]
Palmitic acid (16:0)	>1000
6-Heptadecynoic acid (17:1)[b]	>1000
6-Icosynoic acid (20:1)[b]	>1000
2-Pentacosynoic acid[c]	566 ± 1
5-Pentacosynoic acid (**1a**)	24 ± 1
9-Pentacosynoic acid (**1b**)	>1000
(5*Z*)-5-Pentacosenoic acid (**2a**)	38 ± 1
(9*Z*)-9-Pentacosenoic acid (**2b**)	54 ± 1
Nervonic acid (NA)	12 ± 1
Pentacosanoic acid (25:0)	>1000
2 % DMSO	>1000

[a] RT inhibitory assays were performed in duplicate. The IC_{50} values were calculated using Prism Software (Graphpad, San Diego, CA) from titration curves generated from the experimental values

[b] The synthesis of the shorter-chain Δ^6 acetylenic fatty acids was previously described [53]

[c] Obtained from the reaction of 1-tetracosyne with CO_2 using *n*-BuLi in THF

In the case of the monoenoic fatty acids **2a** and **2b**, changing the position of the double bonds from Δ5 to Δ9 in the C_{25} acyl chain had only some minor effect on the inhibitory activity towards HIV-RT (Table 1). For example, acid **2a** displayed an IC_{50} of 38 ± 1 μM against HIV-1 RT, while acid **2b** displayed an IC_{50} of 54 ± 1 μM. It seems that the olefinic acid **2b** sits better into the HIV-RT active site than the acetylenic acid **1b**, being the *cis* double bond stereochemistry critical for the inhibition.

It is known that the degree of unsaturation in fatty acids affects its inhibitory activity towards DNA polymerase enzymes, such as the DNA polymerase β [33–35]. Since HIV-1 RT shares structural similarities with the DNA pol β enzyme [36], this would imply, as we have shown, that the unsaturation should also affect the inhibitory activity of fatty acids towards the RT enzyme. Our results do reveal a dependency between the degree and site of unsaturation and the inhibitory activity of these fatty acids, e.g., Δ^5-25:1 versus 25:0. Moreover, the position of the triple bond unsaturation was also important for the inhibition since a triple bond at C-5 was more effective than a triple bond at either C-2 or C-9. Our results also show that the longer the fatty acid chain length (C_{25} vs. either C_{20} or C_{17}) the greater its inhibitory activity. This coincides with previous studies with the HIV-1 RT as well as with DNA pol β, where the long-chain fatty acids had greater inhibitory activity against these enzymes [33].

Molecular Modeling Studies

To better understand the relationships between fatty acid chain length, degree of unsaturation and inhibitory activity, we modeled the binding modes of acids **1a** and **2a** to the p51 subunit of HIV-1 RT. Both fatty acids tested herein have similar molecular interactions in the HIV-RT binding pocket as those exhibited by nervonic acid [36] (Fig. 1a, b). The carboxylate group of these fatty acids interacts with key amino acids such as Lys65, Lys66, Lys220 and Tyr232, while their long alkyl chain establishes attractive hydrophobic interactions with the side chain of Lys104, Ile195, and His235. The modeling studies also indicated that shorter carbon chains would not be capable of stabilizing the molecule within the binding site. An ideal length of 25 carbons is required to fulfill the binding site, thereby inhibiting the enzymatic activity. Furthermore, the small difference in the inhibitory activities between **1a** and **2a** might be due to the *cis*-configuration of acid **2a** preventing it from

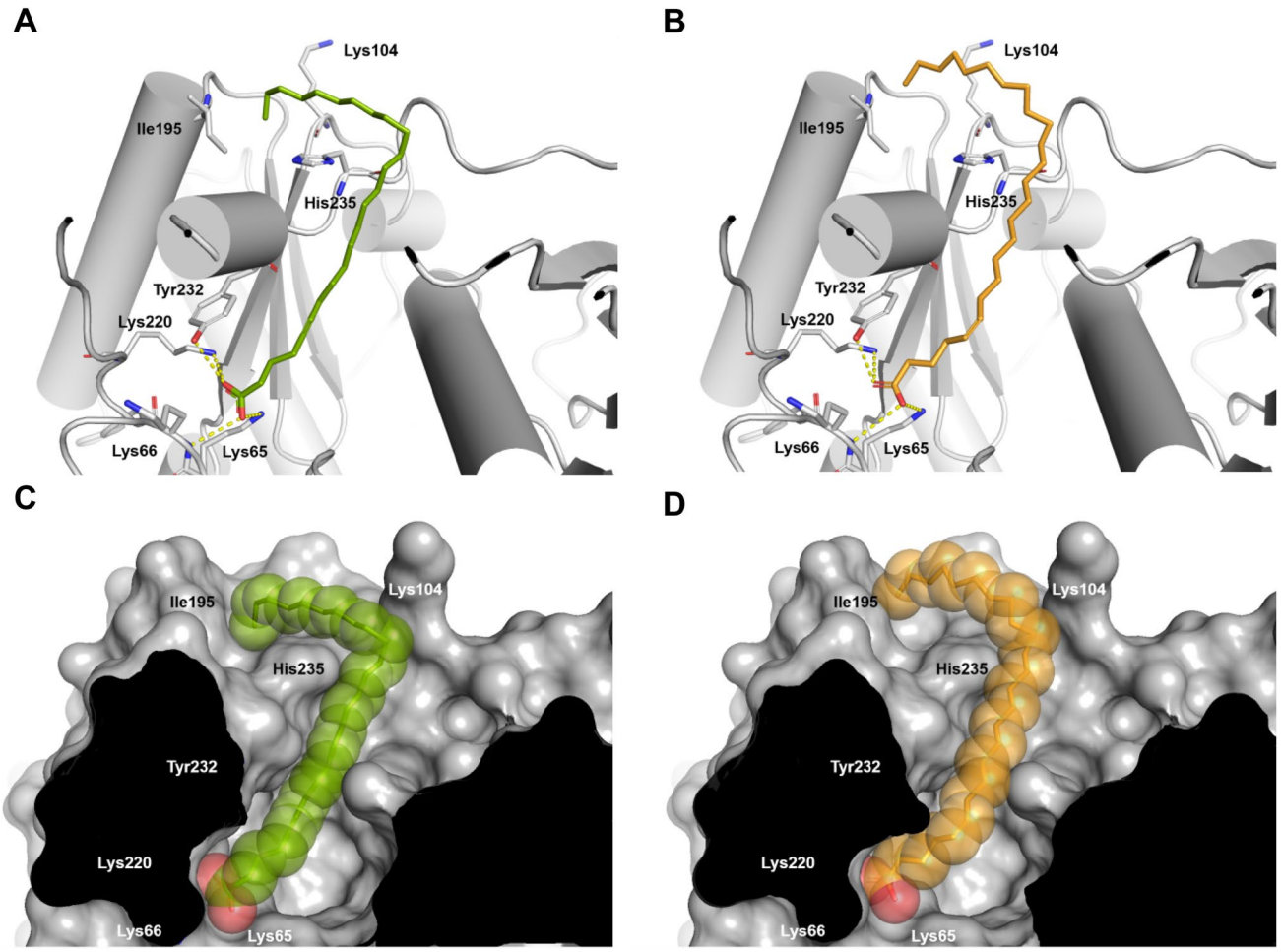


Fig. 1 Modeled binding modes of the fatty acids **1a** (*green*) and **2a** (*orange*) in the p51 subunit of HIV-1 RT. **a**, **b** Inhibitors and residues involved in the ligand-receptor binding are indicated as *stick* models, protein structure is indicated as cartoon and electrostatic interactions as *yellow dashed lines*. **c**, **d** View of the fatty acid binding site indicating the solvent accessible surface of p51 subunit and the spatial complementarity of the inhibitors (*sphere* model) (color figure online)

fully occupying the HIV-RT hydrophobic cavity formed by Lys104, Ile195 and His235 (Fig. 1c, d). It is also important to note that the unsaturations constrain the alkyl chain degrees of freedom, thereby orienting the carbon chain toward the hydrophobic pocket close to Ile195, Lys104 and His235. The latter can explain the fact that pentacosanoic acid does not inhibit HIV-1 RT at all.

Cytotoxicity

The cytotoxicities of **1a, 1b**, **2a**, **2b** as well as nervonic acid were tested against peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers following a methodology previously described by us [52]. In this assay none of the synthetic fatty acids tested, including nervonic acid, displayed significant toxicity at concentrations higher than 500 μM. Comparing these results with the effective dose necessary for HIV-1 RT inhibition, both acids **1a** and **2a** would not be cytotoxic at their optimum inhibitory dose concentrations of 24 and 38 μM, respectively.

Conclusions

In summary, we have shown that **2a**, as well as its acetylenic analogue **1a**, are inhibitors of the HIV-1 RT enzyme. While other modes of action could be envisaged for acids **1a** and **2a**, the one presented herein was consistent with the enzymatic inhibitory studies. These results open the door to the synthesis of other structurally related analogs, which can combine in a single molecule, favorable structural features of both NA and **1a** for a more efficient HIV-RT inhibition. The first total synthesis for the naturally occurring fatty acids **2a** and **2b** was also accomplished.

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