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ORIGINAL RESEARCH

Synthesis, computational evaluation and pharmacological assessment of acetylsalicylic esters as anti-inflammatory agents

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Abstract

A convenient approach to the synthesis of alkyl esters of aspirin (ASA-OR) has been developed. The synthesis of ASA-OR has been realized in two steps: (1) direct esterification of salicylic acid with alcohols in the presence of dicyclohexylcarbodiimide to give alkyl salicylates (SAL-OR); (2) acetylation of SAL-OR with acetyl chloride to yield ASA-OR. Molecular mechanics simulations, performed to calculate the kinetic radii of several ASA-OR, indicated that the pentyl and hexyl acetylsalicylates possess the best properties to cross cell membranes. The in vitro biological tests demonstrate their anti-inflammatory activity, superimposable to that of aspirin. The results of our study suggest that ASA-OR may be used as anti-inflammatory drugs for topical application.

Keywords Acetylsalicylic acid · Acetylsalicylic esters · Anti-inflammatory activity · Aspirin · Computational study

Introduction

Aspirin (acetylsalicylic acid, ASA) is one of the most known and used non-steroidal anti-inflammatory drugs (Desborough and Keeling 2017). It acts by irreversibly inhibiting both cyclooxygenase (COX) enzymes. ASA acetylates the serine 530 moiety of COX-1, and the serine 516 moiety of COX-2 (Loll et al. 1995), being 170 times more effective in inhibiting the former (Vane et al. 1998). The blockage of these enzymes leads to a decrease of the production of prostaglandins and thromboxane-A2, responsible for its anti-inflammatory and anti-platelet effects, respectively. Despite its significant activity against different conditions, inflammatory and cardiovascular diseases, fever and pain, ASA also presents several important collateral effects, mainly on the gastrointestinal apparatus (Lavie et al. 2017). In order to reduce them, transdermal delivery offers an alternative route for administering ASA that bypasses the gut and may be a more convenient, safer and non-invasive mean for its delivery especially in case of long-term use. For the topic treatment of pain or inflammatory diseases, the use of a formulation based on a slow and low-dose transdermal delivery of ASA would be highly desirable (Shamsher et al. 2010). To avoid its rapid hydrolysis to salicylic acid (SAL) during skin absorbtion, it has been suggested to use more stable aspirin derivatives, such as salicylic esters ASA-OR (R = alkyl), able to efficiently cross the skin and then release the more hydrophilic ASA subcutaneously by hydrolysis (McMahon et al. 1988).

Some alkyl esters of ASA were previously prepared by esterification of acetylsalicyloyl chloride with the corresponding alcohols, yet in low to modest yields (7–22%) (Gerber et al. 2006). In this work, we have synthesized four salicylic esters ASA-OR **1**–**4**, with R = pentyl (**1**), hexyl (**2**),

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decyl (**3**), and tetradecyl (**4**), by a new and convenient procedure, based on two synthetic steps: (1) direct esterification of SAL with aliphatic alcohols (ROH), in the presence of dicyclohexylcarbodiimide (DCC), to give alkyl salicylates (SAL-OR); (2) acetylation of SAL-OR with acetyl chloride to yield ASA-OR. We have also performed molecular mechanics simulations to evaluate whether these agents are able to cross cellular membranes and assessed their anti-inflammatory activity in vitro.

Materials and methods

Synthesis of alkyl acetylsalicylates (ASA-OR) 1–4

General

Anhydrous THF was purchased from Sigma Aldrich (Sigma-Aldrich Italia s.r.l., Milano, Italy). All other solvents and chemicals were reagent grade and used without further purification (Sigma-Aldrich). Starting material, salicylic acid, was commercially available (Sigma-Aldrich). All reactions were analyzed by TLC on silica gel 60F254 (Merck) and by GLC by using a Shimadzu GC-2010 gas chromatograph and capillary columns with polymethylsilicone + 5% phenylsilicone (HP-5) as the stationary phase. Evaporation refers to the removal of solvent under reduced pressure. Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 25 °C with a Bruker DPX Avance 300 Spectrometer in $CDCl_3$ solutions at 300 and 75 MHz, respectively, with Me_4Si as internal standard. Chemical shifts (δ) and coupling constants (J) are given in ppm and in Hz, respectively. IR spectra were taken with a JASCO FT-IR 4200 spectrometer. Mass spectra were obtained by using a Shimadzu QP-2010 GC-MS apparatus at 70 eV ionization voltage and by electrospray ionization mass spectrometry (ESI-MS) by using an Agilent 6540 UHD accurate-mass Q-TOF spectrometer equipped with a Dual AJS ESI source working in positive or negative mode.

Esterification of salicylic acid (SAL) to give alkyl salicylates (SAL-OR)

To a solution of SAL (500 mg, 3.62 mmol) in anhydrous THF (200 mL), maintained at 0 °C and under nitrogen, was added the alcohol ROH (5.43 mmol; 1.5 equiv) (R = pentyl: 480 mg; hexyl: 555 mg; decyl: 860 mg, tetradecyl: 1.17 g). To the resulting mixture was added, dropwise and under nitrogen, a solution of DCC (1.57 g, 7.61 mmol) in anhydrous THF (200 mL). After stirring at room temperature for 20 h, the mixture was concentrated under vacuum and filtered to remove the excess DCC. The solid was washed with AcOEt, and the solution was first acidified with 1 M HCl and then neutralized with saturated $NaHCO_3$. The organic layer was washed with water (3 × 50 mL) and then dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude product was purified by column chromatography on silica gel, using hexane-AcOEt 99:1 as eluent.

Pentyl salicylate Yield: 300 mg, starting from 500 mg of SAL (40%). Yellow oil. IR (film): $\nu = 3183$ (w, br), 1676 (s), 1327 (m), 1302 (m), 1214 (m), 1158 (m), 757 (m), 701 (w); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 10.87 (s, 1H, OH), 7.85 (dd, $J = 8.0$, 1.7, 1H, aromatic), 7.49–7.40 (m, 1H, aromatic), 6.94 (d, $J = 8.4$, 1H, aromatic), 6.92–6.88 (m, 1H, aromatic), 4.34 (t, $J = 6.7$, 2H, OCH_2), 1.87–1.72 (m, 2H, $OCH_2CH_2CH_2$), 1.51–1.33 (m, 4H, $CH_2CH_2CH_3$), 0.94 (t, $J = 7.1$, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 170.3, 161.7, 135.6, 129.9, 119.1, 117.6, 112.6, 65.5, 28.3, 28.1, 22.3, 14.0; GC-MS: $m/z = 208$ (21) [M^+], 138 (31), 120 (100), 92 (13), 65 (8). HRMS-ESI [$(M\text{-}H)^-$]: m/z calcd for $(C_{12}H_{15}O_3)^-$: 207.1027; found, 207.1035.

Hexyl salicylate Yield: 325 mg, starting from 500 mg of SAL (40%). Yellow oil. IR (film): $\nu = 3184$ (m, br), 1676 (s), 1586 (w), 1302 (m), 1214 (m), 1091 (m), 757 (m), 701 (w); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 10.87 (s, 1H, OH), 7.83 (dd, $J = 8.0$, 1.7, 1H, aromatic), 7.47–7.37 (m, 1H, aromatic), 6.96 (dd, $J = 8.5$, 1.7, 1H, aromatic), 6.90–6.81 (m, 1H, aromatic), 4.32 (t, $J = 6.7$, 2H, OCH_2), 1.76 (q, $J = 6.8$, 2H, $OCH_2CH_2CH_2$), 1.51–1.21 [m, 6H, $(CH_2)_3CH_3$], 0.99–0.81 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 170.3, 161.7, 135.5, 129.9, 119.1, 117.6, 112.7, 65.5, 31.5, 28.6, 25.7, 22.6, 14.0; GC-MS: $m/z = 222$ (10) [M^+], 138 (47), 120 (100), 92 (15). The spectroscopic properties agreed with those reported (Chighine et al. 2009).

Decyl salicylate Yield: 458 mg, starting from 500 mg of SAL (45%). Yellow oil. IR (film), ν: 3190 (m, br), 1675 (s), 1302 (m), 1198 (m), 1158 (m), 757 (m), 701 (w); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 10.87 (s, 1H, OH), 7.83 (dd, $J = 8.0$, 1.5, 1H, aromatic), 7.48–7.38 (m, 1H, aromatic), 6.96 (d, $J = 8.4$, 1H, aromatic), 6.90–6.82 (m, 1H, aromatic), 4.33 (t, $J = 6.7$, 2H, OCH_2), 1.85–1.69 (m, 2H, $OCH_2CH_2CH_2$), 1.52–1.15 [m, 14H, $(CH_2)_7CH_3$], 0.94–0.83 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 170.3, 161.7, 135.5, 129.9, 119.1, 117.6, 112.7, 65.5, 31.9, 29.58, 29.56, 29.35, 29.28, 28.6, 26.0, 22.7, 14.1; GC-MS: $m/z = 278$ (31) [M^+], 138 (89), 120 (100), 92 (11). HRMS-ESI [$(M\text{-}H)^-$]: m/z calcd for $(C_{17}H_{25}O_3)^-$: 277.1809; found, 277.1793. The spectroscopic properties agreed with those reported (Kalbasi et al. 2010).

Tetradecyl salicylate Yield: 489 mg, starting from 500 mg of SAL (40%). Yellow solid, m.p. 37–38 °C. IR (KBr): v = 3125 (w, br), 1673 (s), 1470 (m), 1303 (m), 1215 (m), 1157 (m), 762, 668 (w); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 10.87 (s, 1H, OH), 7.84 (d, J = 7.8, 1H, aromatic), 7.50–7.36 (m, 1H, aromatic), 6.97 (d, J = 8.4, 1H, aromatic), 6.92–6.80 (m, 1H, aromatic), 4.33 (t, J = 6.6, 2H, OCH_2), 1.86–1.70 (m, 2H, $OCH_2CH_2CH_2$), 1.50–1.05 [m, 22H, $(CH_2)_{11}CH_3$], 0.94–0.79 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 170.3, 161.7, 135.5, 129.9, 119.1, 117.6, 112.7, 65.5, 31.9, 29.72, 29.71, 29.69, 29.67, 29.61, 29.54, 29.39, 29.26, 28.6, 26.0, 22.7, 14.1; GC-MS: m/z = 334 (37) [M^+], 138 (100), 120 (80), 92 (8). HRMS-ESI [$(M\text{-}H)^-$]: m/z calcd for $(C_{21}H_{33}O_3)^-$: 333.2435; found, 333.2445.

Acetylation of alkyl salicylates to give alkyl acetylsalicylates ASA-OR 1–4

To a solution of the alkyl salicylate (1.44 mmol) (R = pentyl: 300 mg; hexyl: 320 mg; decyl: 400 mg, tetradecyl: 482 mg) in anhydrous CH_2Cl_2 (13 mL), was added, at room temperature and under nitrogen, Et_3N (365 mg, 3.6 mmol) followed by AcCl (290 mg, 3.7 mmol). After stirring at 40 °C for 20 h, satd NH_4Cl was added (5 mL). The resulting mixture was extracted at room temperature with CH_2Cl_2 (3 × 15 mL), and the collected organic layers dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude product was purified by column chromatography on silica gel, using hexane-AcOEt99:1 as eluent.

Pentyl acetylsalicylate (1) Yield: 163 mg, starting from 300 mg of pentyl salicylate (45%). Yellow oil. IR (film): v = 1778 (s), 1723 (s), 1294 (m), 1196 (s), 1160 (s), 1083 (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.03 (dd, J = 7.8, 1.7, 1H, aromatic), 7.60–7.50 (m, 1H, aromatic), 7.36–7.27 (m, 1H, aromatic), 7.10 (dd, J = 8.0, 0.7, 1H, aromatic), 4.27 (t, J = 6.8, 2H, OCH_2), 2.35 (s, 3H, COMe), 1.82–1.66 (m, 2H, $OCH_2CH_2CH_2$), 1.46–1.31 (m, 4H, $CH_2CH_2CH_3$), 0.97–0.86 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 169.7, 164.5, 150.6, 133.7, 131.7, 126.0, 123.8, 123.5, 65.3, 28.3, 28.1, 22.3, 21.0, 13.9; HRMS-ESI [$(M+Na)^+$]: m/z calcd for $(C_{14}H_{18}NaO_4)^+$: 273.1077; found, 273.1080.

Hexyl acetylsalicylate (2) Yield: 252 mg, starting from 320 mg of hexyl salicylate (66%). Yellow oil. IR (film): v = 1772 (s), 1723 (s), 1294 (m), 1196 (s), 1083 (m), 915 (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.02 (d, J = 7.8, 1H, aromatic), 7.61–7.49 (m, 1H, aromatic), 7.36–7.24 (m, 1H, aromatic), 7.09 (d, J = 8.1, 1H, aromatic), 4.26 (t, J = 6.7, 2H, OCH_2), 2.35 (s, 3H, COMe), 1.81–1.62 (m, 2H, $OCH_2CH_2CH_2$), 1.52–1.20 [m, 6H, $(CH_2)_3CH_3$], 0.98–0.80 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 169.7, 164.6, 150.7, 133.7, 131.7, 126.0, 123.8, 123.5, 65.3, 31.5, 28.6, 25.6, 22.5, 21.0, 14.0; HRMS-ESI [$(M+Na)^+$]: m/z calcd for $(C_{15}H_{20}NaO_4)^+$: 287.1254; found, 287.1234.

Decyl acetylsalicylate (3) Yield: 208 mg, starting from 400 mg of decyl salicylate (45%). Yellow solid, m.p. 41–42 °C. IR (KBr): v = 1776 (s), 1720 (s),1606 (m), 1373 (m), 1256 (s), 1200 (m), 1081 (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.02 (dd, J = 7.8, 1.6, 1H, aromatic), 7.59–7.50 (m, 1H, aromatic), 7.35–7.27 (m, 1H, aromatic), 7.10 (dd, J = 8.0, 1.1, 1H, aromatic), 4.26 (t, J = 6.7, 2H, OCH_2), 2.35 (s, 3H, COMe), 1.82–1.66 (m, 2H, $OCH_2CH_2CH_2$), 1.47–1.18 [m, 14H, $(CH_2)_7CH_3$], 0.94–0.83 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 169.7, 164.6, 150.7, 133.7, 131.7, 126.0, 123.8, 123.5, 65.3, 31.9, 29.54, 29.52, 29.30, 29.28, 28.6, 26.0, 22.7, 21.0, 14.1; HRMS-ESI [$(M+Na)^+$]: m/z calcd for $(C_{19}H_{28}NaO_4)^+$: 343.1880; found, 343.1857.

Tetradecyl acetylsalicylate (4) Yield: 300 mg, starting from 482 mg of tetradecyl salicylate (55%). Yellow solid, m.p. 39–40 °C. IR (KBr): v = 1766 (s), 1720 (s), 1255 (s), 1201 (m), 1084 (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.06–7.97 (m, 1H, aromatic), 7.61–7.49 (m, 1H, aromatic), 7.36–7.25 (m, 1H, aromatic), 7.13–7.06 (m, 1H, aromatic), 4.26 (t, J = 6.8, 2H, OCH_2), 2.36 (s, 3H, COMe), 1.87–1.61 (m, 2H, $OCH_2CH_2CH_2$), 1.58–1.02 [m, 22H, $(CH_2)_{11}CH_3$], 1.01–0.79 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 169.7, 164.5, 150.7, 133.7, 131.7, 126.0, 123.8, 123.5, 65.3, 31.9, 29.69, 29.66, 29.60, 29.5, 29.4, 29.3, 28.6, 26.0, 22.7, 21.0, 14.1; HRMS-ESI [$(M+Na)^+$]: m/z calcd for $(C_{23}H_{36}NaO_4)^+$: 399.2506; found, 399.2479.

Computational approach

The ASA-OR conformational analysis was performed by Molecular Mechanics using the universal force field and Avogadro code (Hanwell et al. 2012), which is an application of the Open Babel platform (O'Boyle et al. 2011). A search method converging to the low energy conformers, by weighing torsion angles as function of the total energy, was employed. For each acetylsalicylic esters, a sample of 30 × 10^4 conformers was analyzed and the weighed rotor search was applied to find the more stable conformations. Finally, the most stable fifty structures were used for the calculation of the kinetic radii, after a last geometry optimization using a conjugate gradient algorithm.

Cell culture and cytotoxicity study

Cytotoxic studies

Experiments were carried out on the human leukemia monocytic cell line THP-1, originally obtained from ATCC (Rockville, MD, USA). The cultures were grown according to the literature (Currò et al. 2016). Stock solutions of each tested compound (ASA, **1**, **2**, and **3**) were prepared in dimethylsulfoxide (DMSO) at 100 mM concentration and stored in aliquot at −20 °C. Compound **4** was insoluble in DMSO, thus not allowing us to test its biological activity. Products were diluted in culture media to the desired concentration just prior the use. The same DMSO concentrations used to dissolve the molecules served as vehicle controls. First, in order to assess the potential cytotoxicity of molecules used in this study, preliminary experiments were carried out on THP-1 cells using both 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Navarra et al. 2015) and lactate dehydrogenase (LDH) test (Romeo et al. 2014a). The former measures the mitochondrial activity of living cells, providing indirect information on cell viability. The latter determines the release of the cytosolic enzyme lactate dehydrogenase into the culture medium when the cell plasma membrane is damaged (dead cells). In both tests, THP-1 cells were incubated in 96-well culture plates at a density of 5×10^3 cells/well with fresh red-phenol free medium containing increasing concentrations of molecules (ASA, **1**, **2**, and **3**) at 37 °C for 24 h. Then, for MTT assay, the plates were centrifuged, the supernatants were replaced with 100 µL of media containing 0.5 mg/mL of MTT (Sigma-Aldrich, Milan, Italy), and the plates were returned in the incubator for 4 h. After a centrifugation, the supernatants were removed and the crystals of formazan (MTT metabolic product) were solubilized with 100 µL HCl/isopropanol 0.1 N lysis buffer. Absorbance was determined at 570 nm with reference at 690 nm, using a microplate reader (iMark™ microplate reader). Results are expressed as percentages of MTT reduction respect to control cultures. All experiments were performed in eight replicates and repeated three times (Romeo et al. 2014b).

In LDH assay, we used a commercial LDH kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega), according to the manufacturer's protocol. Briefly, plates were centrifuged at $400 \times g$ for 5 min, and 100 µL of supernatant from each well was transferred to corresponding wells on a new plate. Then, freshly prepared 100 µL LDH reaction solution was added to each well, and the plate placed on an orbital shaker for 30 min at room temperature. Absorbance at 490 nm was quantified spectrophotometrically. LDH levels are extrapolated as the values detected in control cells, which are arbitrarily expressed as 1. All the experiments were carried out in triplicate and repeated three times (Romeo et al. 2014a).

Real-time PCR

The experimental model used to evaluate the anti-inflammatory activity of the molecules **1**, **2**, and **3** consisted in THP-1 cells stimulated with lipopolysaccharide (LPS; 500 ng/ml) for 3 h or 6 h (Risitano et al. 2014). Cells (1×10^6) were seeded into six/wells plates and the next day were treated or not with 100 µM ASA, **1**, **2**, or **3**, prior to add LPS (30 min). Extraction of total cellular RNA and cDNA synthesis were performed as described (Ferlazzo et al. 2016) Briefly, after RNA isolation with TRIzol reagent (Invitrogen Life Technologies, Milan, Italy), RNA (2 µg) was reverse transcribed with High Capacity cDNA Archive kit (Applied Biosystems, Applera Corp., Milan, Italy) according to the manufacturer's instructions. Then, mRNA levels of IL-1β, TNF-α, cyclooxygenase-1 and 2 (COX-1 and COX-2), and inducible nitricoxide synthase (iNOS) were analyzed by real-time PCR. β-Actin mRNA was used as endogenous control. Quantitative PCR reactions were set up in triplicate in a 96-well plate and were carried out in 20 µL reactions containing 1xSYBR® Select Master Mix (Applied Biosystems), 0.2 µM of each primer (they are listed in Table 1) and 25 ng RNA converted into cDNA. The analyses were performed in a 7500 real-time PCR System (Applied Biosystems) and the data were collected and analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method.

Results and discussion

Synthesis of acetylsalicylic esters (ASA-OR) 1–4

As said in the Introduction, some alkyl acetylsalicylates ASA-OR (R = Me, Et, Pr, *i*-Pr, Bu, 1-methylpropyl, *t*-Bu, pentyl, 1-methylbutyl, and 1-ethylpropyl) were previously

Table 1 Primers used for real-time PCR

Gene product	Primers sequence
COX1	Forward: 5′ -CTTGTGCTCCTCTTGATT- 3′
	Reverse: 5′ -CACTCCACCATTCTGTCT- 3′
COX2	Forward: 5′ -GCCTGGTCTGATGATGTA - 3′
	Reverse: 5′ -TCTGGAACAACTGCTCAT - 3′
iNOS	Forward: 5′ -GGATGACAACCGATACCA - 3′
	Reverse: 5′ -GAAGGCAATGGACTCAGA - 3′
IL1-β	Forward: 5′ -GCTTATTACAGTGGCAATGA - 3′
	Reverse: 5′ -TAGTGGTGGTCGGAGATT - 3′
TNF-α	Forward:5′ -GTGAGGAGGACGAACATC - 3′
	Reverse: 5′ -GAGCCAGAAGAGGTTGAG - 3′
β-Actin	Forward: 5′ -TTGTTACAGGAAGTCCCTTGCC - 3′
	Reverse: 5′ -ATGCTATCACCTCCCCTGTGTG - 3′

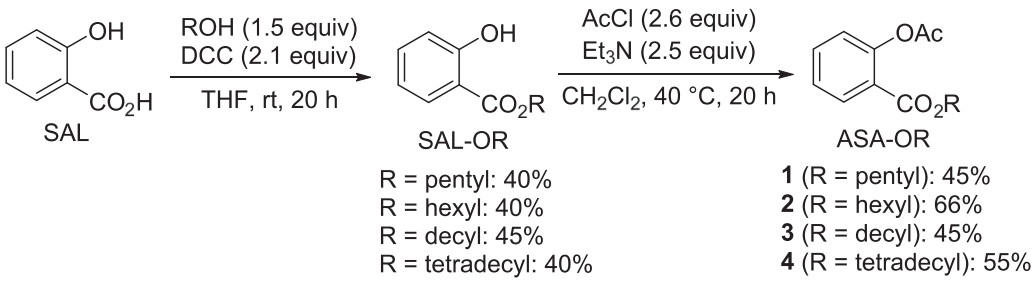

Fig. 1 Synthesis of acetylsalicylic esters (ASA-OR) **1**–**4** by direct esterification of salicylic acid (SAL) to give alkyl salicylates (SAL-OR) followed by acetylation

prepared in 7–22% yield by esterification of acetylsalicyloyl chloride with the corresponding alcohols (Gerber et al. 2006). We present here an alternative and convenient approach to alkyl acetylsalicylates (with R = pentyl, hexyl, decyl, and tetradecyl, in particular), based on the direct esterification of salicylic acid with alcohols ROH in the presence of DCC followed by acetylation with acetyl chloride, according to the scheme shown in Fig. 1.

As can be seen from the Scheme, fair isolated yields of the final alkyl acetylsalicylates **1**–**4** (18–26% over the two steps) were obtained under particularly mild conditions and starting from readily available substrates.

Computational analysis of acetylsalicylic esters kinetic radii

Four permeation pathways can be considered to describe the passage of organic compounds across the cute: (a) diffusion through the double lipid layers of the cellular membrane (due to the presence of free volumes); (b) solute transport (ensuing from lateral diffusion along lipid layers; for molecules with Mw > 400 Da and partition constant $K_{o/w} > 1$); (c) diffusion through pores (for small molecules with $K_{o/w} < 0.01$; and (d) diffusion through hair follicles and sweat glands (for compounds with Mw > 10,000 and $K_{o/w} < 0.01$).

In the case of small and hydrophobic solutes, with Mw < 400 Da and $K_{o/w} > 1$, the permeation is mainly controlled by the diffusion through the free volumes of the double lipid layers. The ASA-OR, theoretically investigated in this work (R = butyl, pentyl, 2-methylpentyl, hexyl, 2-methylhexyl) possess molecular masses < 400 Da and the partition coefficients for acetates ROAc are all >1. Thus, the diffusion through the double lipid layers should be the predominant permeation mechanism with respect to the others. The diffusion coefficient can be evaluated as function of the solutes partition constant, $K_{o/w}$ and kinetic radius, r_{kic}, by means of the following model equation (Mitragotri 2003):

$$K_p^{fv}\left(r_{kic}, K_{o/w}\right) = 5,6 \times 10^{-6} K_{o/w}^{0,7} \exp\left(-0,46 r_{kic}^2\right) \qquad (1)$$

Table 2 Minimum (r^{inf}_{kic}) and maximum (r^{sup}_{kic}) kinetic radii of five ASA-OR (in brackets the corresponding % of isomers)

Acetylsalicylic esters (ASA-OR)	r^{inf}_{kic} (Å) and % isomer	r^{sup}_{kic} (Å) and % isomer	% Isomer with 5 Å < r_{kic} < 6 Å	Log $K_{o/w}$ [a]
R = butyl	5 (10)	8 (5)	63	1.8
R = pentyl	5 (5)	9 (1)	61	1.9
R = 2-metylpentyl	6 (15)	9 (5)	15	
R = hexyl	6 (33)	9 (5)	33	2.4
R = 2-metylhexyl	6 (50)	9 (10)	50	

[a]Values for acetates ROAc (Abraham et al. 1994)

Therefore, the diffusion of ASA-OR exponentially decreases as their kinetic radius increases, and increses as the partition constants rise (Mitragotri 2003). According to Eq. (1), the kinetic radius of the most stable ASA-OR conformers provides information on the diffusion of molecules having similar $K_{o/w}$. Different empirical formulas are available in the literature to calculate the kinetic radii as function of the molecular weights. Clearly, each ASA-OR shows a huge number of conformers, so these empirical formulas are not directly applicable in our case. For this reason, in this work, the r_{kic} of the acetylsalicylic esters were calculated using a previously validated algorithm (Buekenhoudt et al. 2013), which takes into account the elevated number of conformers. In particular, the kinetic radii of the 50 more stable conformers, selected from a sample of 30×10^4 conformers were calculated. From this calculation, it was possible to determine the minimum and maximum kinetic radii, that are, r^{inf}_{kic} and r^{sup}_{kic}, respectively, and the percentage of ASA-OR conformers having r_{kic} between 5 Å and 6 Å; the results are shown in Table 2.

All the stable conformers of the investigated ASA-OR show r_{kic} higher than 5 Å. The range $r^{inf}_{kic} - r^{sup}_{kic}$ remains substantially constant as the number of the carbon atoms in the chain of the alkyl chain increases. This means that the diffusion of the ASA-OR is mainly controlled by $K_{o/w}^{0,7}$. From Table 2, it can be seen that log$K_{o/w}$ increases with the

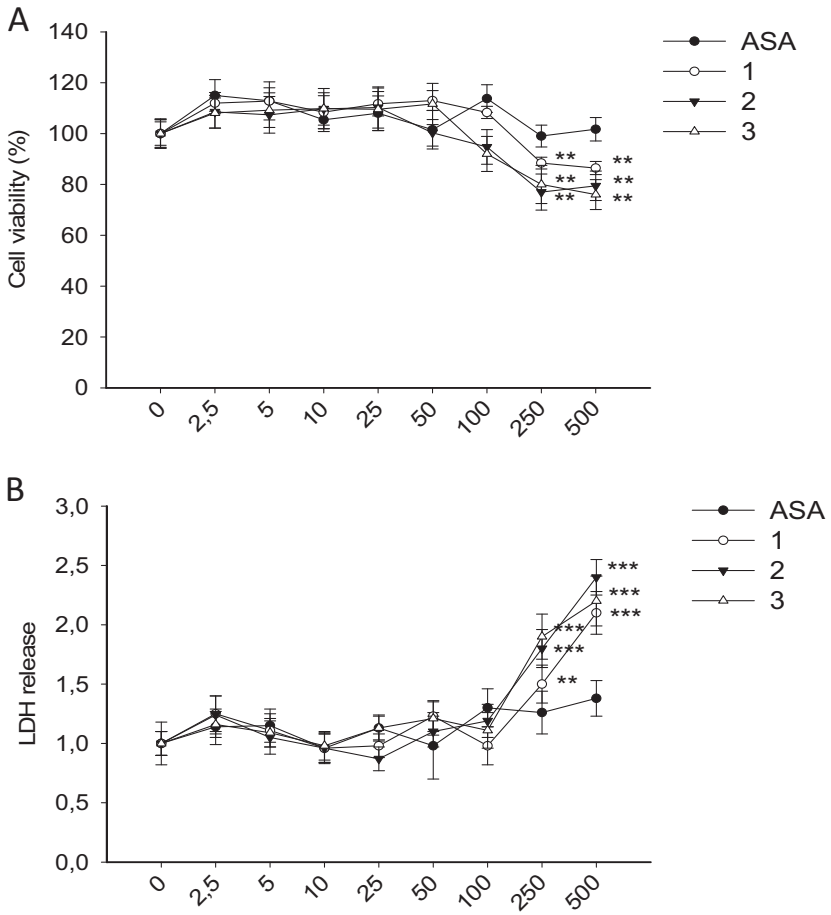

Fig. 2 Effects of ASA, **1**, **2**, and **3** on cell proliferation and LDH release. THP-1 cells were exposed to increased concentration (μM) of compounds for 24 h. **a** Proliferation rate assessed by MTT assay. **b** Cytotoxic effect assessed in terms of LDH release after 24 h of exposure. Each value is the mean ± S.E.M. of three experiments performed eight times (MTT) or in triplicate (LDH) and repeated three different times. $^{**}p < 0.01$ vs. ctrl, $^{***}p < 0.001$ vs. ctrl

length of the alkyl chain, therefore acetylsalicylic esters with longer R chains should permeate more easily as long as the ($r^{\text{inf}}_{\text{kic}} - r^{\text{sup}}_{\text{kic}}$) range remains constant. Furthermore, the distribution of the kinetic radius in this range is another important feature to consider. In fact, comparing the kinetic radii of the ASA-OR with R = pentyl and hexyl, the 56% of the conformers with R = pentyl shows r_{kic} equal to 6 Å, while only 33% of the conformers with R = hexyl displays an equal kinetic radius. This means that the number of the ASA-OR conformers with R = pentyl, which possess a r_{kic} equal to 6 Å, is greater than the number of the ASA-OR conformers with R = hexyl. Since the $\log K_{\text{o/w}}$ of these ASA-OR are similar, the derivative with R = pentyl should show a slightly greater diffusion compared to the derivative with R = hexyl derivative. On the other hand, the ASA-OR with R = butyl should show a similar permeability with respect to the ester R = pentyl. The 50% of the more stable conformers of ASA-OR with R = 2-metylhexyl shows a kinetic radius around 6 Å; as a result, they should show a permeability similar to the derivative with R = pentyl. Moreover, the ASA-OR with R = 2-metylpentyl has only the 15% of its conformers with r_{kic} equal to 6 Å, which means that this ASA-OR should permeate the double lipid layers less effectively than ASA-OR with R = pentyl.

Biological experiments on acetylsalicylic esters (ASA-OR) 1–3

In order to find the concentration of the molecules **1**, **2**, and **3** that did not induce cytotoxicity, preliminary experiments were carried out using both MTT and LDH tests. Exposure of THP-1 monocytes to different concentrations of tested compounds (in a range 2.5–100 μM) for 24 h did not show any significant cytotoxic effects (Fig. 2), while the 250 and 500 μM concentrations produced a significant reduction of cell viability (Fig. 2a), accompanied by cell death (Fig. 2b). Therefore, we chose the 100 μM concentration to detect the anti-inflammatory activity of the molecules **1**, **2**, and **3**. Moreover, based on published data (Risitano et al. 2014), we used LPS at the concentration of 500 ng/mL that has been shown to trigger the release of pro-inflammatory mediators in THP-1 cells within 3 h.

Data of the real-time PCR analyses showed that stimulation of THP-1 monocytes with 500 ng/mL LPS for 3 h produced a dramatic increase of IL-1β, TNF-α and COX-2 mRNA levels, without affecting those of both of COX-1 and iNOS. In particular, in LPS-treated cells, the levels of IL-1β mRNA were more than 1000-fold higher than those found in untreated cells (Fig. 3), as well as those of TNF-α and COX-2 were almost

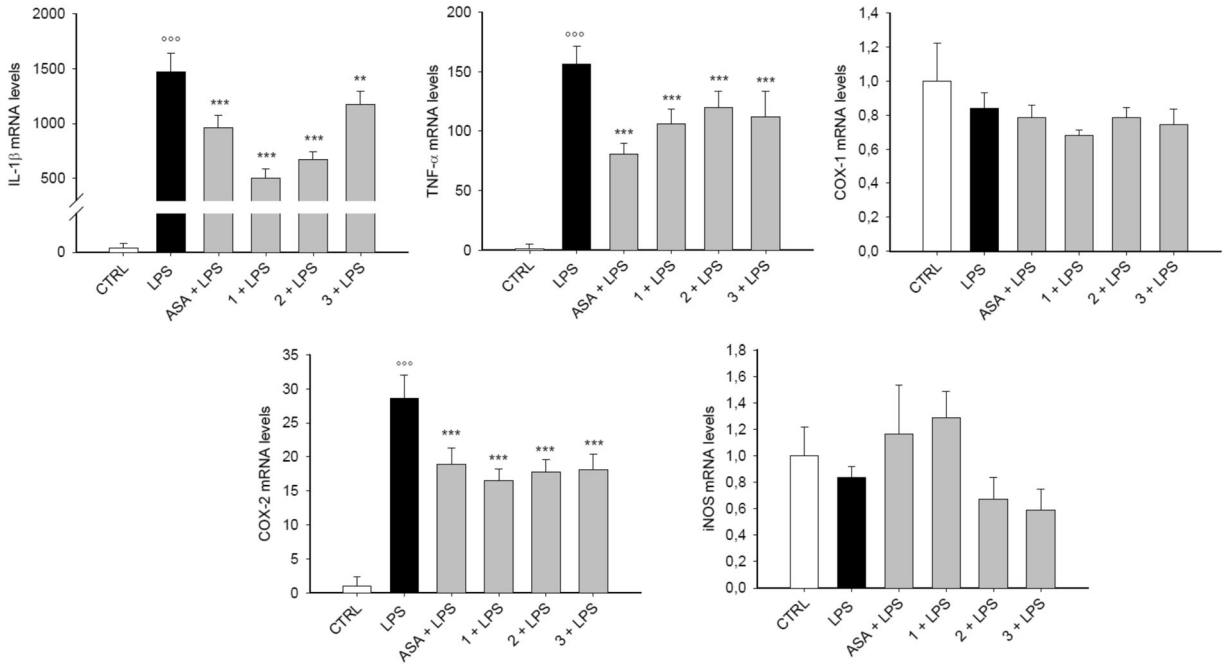

Fig. 3 Effects of ASA and molecules **1**, **2**, and **3** on cytokine gene expression in THP-1 cells stimulated with LPS for 3 h. THP-1 cells were treated with 100 µM of ASA, **1**, **2**, or **3** before exposure to 500 ng/mL of LPS for 3 h. Results from real-time PCR are expressed as relative fold change detected in treated cells compared to the untreated culture, after normalization against β-actin used as endogenous control. Columns and bars represent means ± SEM from triplicate experiments. °°°$p < 0.001$ *versus* control cells; ***$p < 0.001$ *versus* LPS-treated cells (ANOVA followed by Student-Newman Keuls multiple comparisons test)

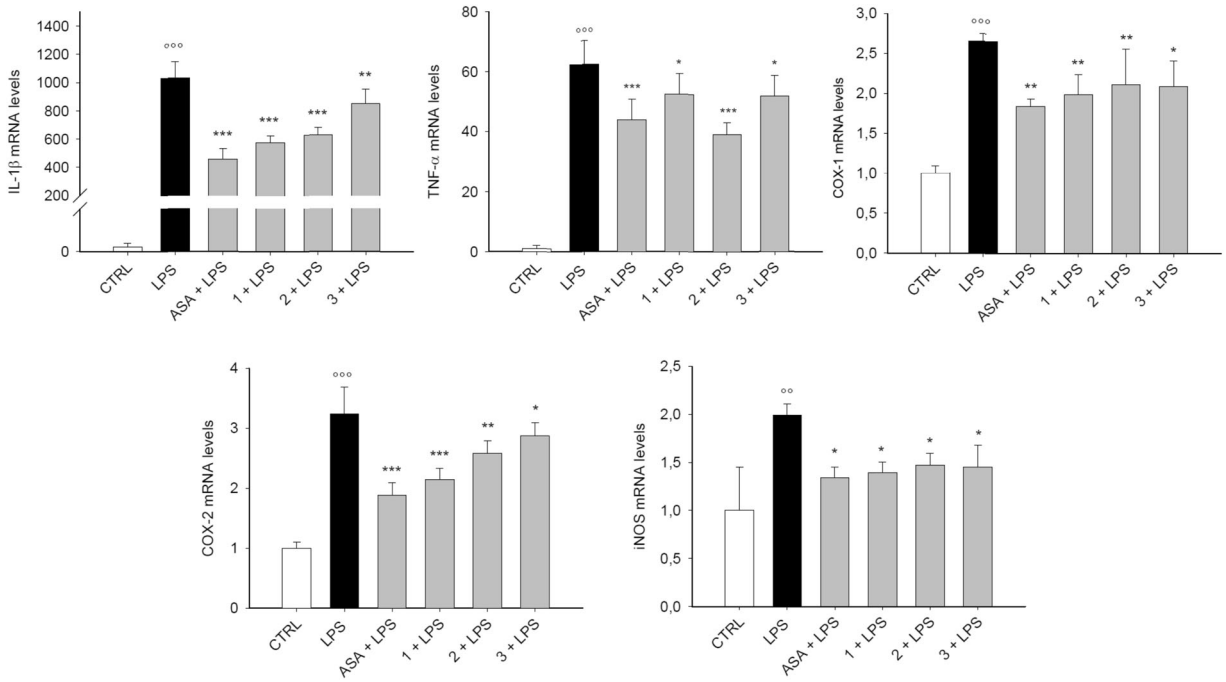

Fig. 4 Real-time PCR of cytokine gene expression at 6 h following LPS exposure. The cells were treated with ASA, **1**, **2** or **3** compound 100 µM prior to be exposed to 500 ng/mL of LPS for 6 h. The experimental results are expressed as relative fold change detected in treated cells compared to the untreated culture, after normalization to β-actin used as endogenous control. Columns and bars represent means ± SEM of three independent experiments. °°°$p < 0.001$ and °°$p < 0.01$ *versus* control cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* LPS-treated cells (ANOVA followed by Student-Newman Keuls multiple comparisons test)

150-fold and about 28-fold higher, respectively, than those in control cells. These effects were decreased in presence of ASA or the **1**, **2**, and **3** molecules. Specifically, the increase of LPS-induced IL-1β mRNA levels were significantly reduced by 100 μM ASA, **1**, **2** or **3** compounds up to 35%, 65%, 55% and 20%, respectively. Similarly, the pre-treatment with ASA, **1**, **2**, or **3** molecules significantly diminished the rise of TNF-α mRNA caused by LPS by 50%, 30%, 25% and 30%, respectively, as well as reduced the COX-2 up-regulation by 35%, 43%, 35% and 30% (Fig. 3). After LPS stimulation for 6 h, the mRNA levels of IL-1β, TNF-α and COX-2 showed similar behavior as those observed after 3 h exposure, either in presence or absence of tested molecules (Fig. 4). It is noteworthy that, in the cells exposed to LPS, the mRNA levels of COX-1 and iNOS were more than doubled with respect to untreated cells (Fig. 4). The pre-incubation of THP-1 cells with ASA produced a 30% and 35% reduction of LPS-induced COX-1 and iNOS mRNA levels, respectively, likewise the molecules **1**, **2**, and **3** that decreased COX-1 and iNOS levels by 20–25% and 25–30%, respectively (Fig. 4).

Conclusion

In conclusion, we have developed a convenient new method for the synthesis of ASA-OR (R = alkyl) as potential candidates for the transdermal application of ASA. The newly synthesized alkyl acetylsalicylates **1**, (R = pentyl), **2** (R = hexyl), and **3** (R = decyl) showed anti-inflammatory properties in vitro superimposable to those of ASA. Molecular mechanics simulations, performed to calculate the kinetic radii of several ASA-OR, indicated that the pentyl and hexyl acetylsalicylates possess the best properties to cross cell membranes. Our study reinforces the finding that alkyl esters of aspirin may be used as anti-inflammatory drugs for topical application.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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