Steroids 117 (2017) 29–37

Contents lists available at ScienceDirect

Steroids

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

Uptake and metabolism of fluorescent steroids by mycobacterial cells

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ARTICLE INFO

Article history:
Received 30 April 2016
Received in revised form 29 September 2016
Accepted 1 October 2016
Available online 5 October 2016

Chemical compounds studied in this article:
Nile Red (PubChem CID: 65182)
22-NBD-cholesterol (PubChem CID: 127604)
Menadione sodium bisulfite (PubChem CID: 23665888)

Keywords:
Fluorescent steroids
Mycobacteria
3β-Hydroxysteroid dehydrogenase
Lipid droplets
Fluorescence microscopy
Mass-spectrometry

ABSTRACT

Fluorescent steroids BODIPY-cholesterol (BPCh) and 7-nitrobenzoxadiazole-4-amino-(NBD)-labeled 22-NBD-chelesterol (22NC) as well as synthesized 20-(NBD)-pregn-5-en-3β-ol (20NP) were found to undergo bioconversions by *Mycobacterium tuberculosis* $H_{37}Rv$ and *M. smegmatis* mc^2 155. The major fluorescent products were determined to be 4-en-3-one derivatives of the compounds. Degradation of NBD fluorophore was also detected in the cases of 22NC and 20NP, but neither NBD degradation nor steroidal part modification were observed for the synthesized 3-(NBD)-cholestane. Mycobacterial 3β-hydroxysteroid dehydrogenases were concluded to be responsible for the formation of the 4-en-3-one derivatives. All the compounds tested were found to cause staining both membrane lipids and cytosolic lipid droplets when incubated with mycobacteria in different manner, demonstrating ability of the steroids to reside in the compartments. The findings reveal a potential of the compounds for monitoring of steroid interactions with mycobacteria and provide information for design of new probes for this purpose.

1. Introduction

Mycobacteria are known to uptake and metabolize cholesterol and some other steroids. The processes are thought to be important for survival and virulence of *Mycobacterium tuberculosis* (Mtb) [1–6]. Thus, modified steroids might be useful as either novel anti-tubercular agents or probes for monitoring of steroid interactions with the pathogen. Recently, side-chain fluorinated steroids have been shown to be able to inhibit growth of mycobacteria [7]. An initial step of the steroid core degradation pathway in mycobacteria is the conversion of cholesterol or similar 5-en-3β-ol steroids into their 4-en-3-one derivatives. This step can be catalyzed by a 3β-hydroxysteroid dehydrogenase (3β-HSD; EC 1.1.1.145) or a cholesterol oxidase (CHOX; EC 1.1.3.6). Mtb possesses 3β-HSD (Rv1106c) and a CHOX homologue ChoD (Rv3409c), whereas Msmeg possesses 3β-HSD (MSMEG_5228) and a ChoD-like enzyme (MSMEG_1604). The purified 3β-HSD from Mtb was proved to convert 3β-hydroxysteroids [8], but there were no reports confirming CHOX activity for ChoD or its homologue from Msmeg. Disruption of genes encoding both ChoD and the Msmeg ChoD-like enzyme were reported to have no effect on growth of either Mtb or Msmeg on media containing cholesterol as a sole carbon source [9,10]. 3β-HSD gene disruption had minimal effect on the viability of Msmeg, but might be more critical in the case of Mtb [8]. ChoD was found to be relevant for Mtb's virulence [6,11], whereas 3β-HSD was not reported to influence mycobacterial pathogenicity.

Abbreviations: BPCh, TopFluor® BODIPY-cholesterol, 23-(1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-24-norcholesterol; 22NC, 22-NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnorcholesterol; 20NP, 20-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-pregn-5-en-3β-ol; 3NC, 3-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-5α-cholestane.

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http://dx.doi.org/10.1016/j.steroids.2016.10.001
0039-128X/

On the other hand, known fluorescent steroids 22-NBD-cholesterol (22NC), 25-NBD-cholesterol (25NC) and TopFluor™ BODIPY-cholesterol (BPCh), are known to be powerful probes for studying various biological processes involving cholesterol [12,13]. The compounds were used in studies of pathogenic bacteria such as *Helicobacter pylori* [14], *Leishmania donovani* [15] and *Borrelia burgdorferi* [16]. However, to the best of our knowledge, no peer-review reports on studies investigating interaction of the fluorescent steroids with mycobacteria can be found in the literature. Highlighting the timeliness of such researches, the new fluorescent derivative of cholesterol carbamate has been recently shown to be taken up by Msmeg mc^2 155 [17]. The compound has been found to be stable in the cells unlike natural cholesterol and its distribution in the bacterial cells has not been shown. Using chromatography, mass-spectrometry and fluorescence microscopy, we investigated general bioconversion features and compartmentalization of BPCh, 22NC as well as two newly synthesized 7-nitrobenzoxadiazol-4-amino- (NBD)-labeled steroids 20-(NBD)-pregn-5-en-3β-ol (20NP) and 3-(NBD)-cholestane (3NC) in Msmeg mc^2 155 and Mtb $H_{37}Rv$ cells. The results provide new details about artificial steroids metabolism and distribution in mycobacteria.

2. Materials and methods

2.1. Chemicals

Fluorescent compounds TopFluor® BODIPY-cholesterol (23-(1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene)-24-norcholesterol; BPCh) (Avanti Polar Lipids, Birmingham, AL), 22-NBD-cholesterol (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-23,24-bisnor-5-cholen-3β-ol; 22NC) (Molecular Probes, Eugene, OR) and 25-NBD-cholesterol (25NC), Avanti Polar Lipids, Birmingham, AL, USA) were used. Nile Red (9-(diethylamino)-5H-benzo[a]phenoxazin-5-one), menadione sodium bisulfite, solvents, buffer components and detergents were purchased from (Sigma-Aldrich, St. Louis, MO).

2.2. Synthesis of new fluorescent steroids with NBD-moiety in 20 and 3 positions

20-(NBD)-pregn-5-en-3β-ol (20NP) and 3-(NBD)-5-cholestane (3NC) were synthesized using reductive amination strategies as described [18–22]. Proton nuclear magnetic resonance (^{1}H NMR) spectra were acquired on a Bruker Avance 400 using $CDCl_3$ as solvent. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane as the internal standard. Coupling constants are in hertz (Hz). Spectrophotometric and fluorescent properties of the synthesized compounds were determined using a UV1202 (Shimadzu) spectrophotometer and a SM2203 (Solar) spectrofluorimeter, respectively. Mass-spectrometric tests were performed as described below.

2.2.1. 20-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-pregn-5-en-3β-ol

Pregnenolone (100 mg, 0.316 mmol) and ammonium acetate (500 mg, 6.78 mmol) were mixed with 10 ml of dry argon-saturated methanol and maintained at 40 °C for 1 h and then NaBH$(OAc)_3$ (425 mg, 6.84 mmol) was added in small portions within 1 h. The solution was stirred until the pregnenolone had completely converted. Then, 10 ml of cooled 0.05 M $NaHCO_3$ were mixed with the solution, and after 1 h the mixture was extracted with ethyl acetate (4 × 10 ml). The combined extract was washed with 10 ml of cold water and evaporated to dryness. The dry residue (yield 84%) was characterized to confirm the presence of two isomers of 20-amino-pregn-5-en-3β-ol by TLC as described [21] and electrospray ionization mass-spectrometry ($[M + H]^+$ peaks with *m/z* values of 318). The NBD-derivative was obtained for more hydrophobic 20*R*- (20α-H) isomer [20,21] as described [22] with modifications. Briefly, the substance dissolved in 5 ml of methanol: chloroform (1:3, v:v); NBD-Cl (100 mg, 0.50 mmol) in 1 ml of the same solvent and saturated sodium carbonate (0.5 g) water solution were added subsequently. The mixture was stirred in the dark overnight at room temperature. Then, 10 ml of cold 1 N hydrochloric acid and 40 ml of chloroform were added. The organic phase was concentrated, and the fluorescent products were purified using silica gel-based column chromatography with benzene and ethyl acetate as mobile phases. Dark orange solid was obtained with 12% yield.

^{1}H NMR: (300 MHz, $CDCl_3$) δ = 8.16 ppm (d, 1H, J = 7.5, benzoxadiazole C6-**H**), 7.13 (d, 1H, J = 7.5, benzoxadiazole C5-**H**), 5.37 (dd, 1H, J = 6.2, C6-**H**), 3.25 (m, 1H, C3-**H**), 2.78 (m, 1H, C20-**H**). **ESI-MS**: *m/z* values of 479 ($[M\text{-}H]^-$); 515 ($[M + Cl]^-$), 481 ($[M + H]^+$), 519 ($[M + K]^+$). **EI-MS**: *m/z* values 480 (M^+, 21%), 241 (6%), 221 (22%), 207 (21%), 191 (20%). **UV–vis**: λ_{max} = 470 nm (methanol). **Fluorimetry**: λ_{max} (emission) = 535 nm (methanol).

Additional purification was performed by HPLC using an LC-10AT (Shimadzu) system equipped with the SPD-M10A photodiode array detector and the LiChroCART C18 (250×4 mm, 5 µm) (Merck) column at eluent flow rate 1 ml/min; acetonitrile (A) and water (B) gradient was used according to the following protocol: 0–5 min, 20% A; 5–25 min, 20–100% A; 30–40 min, 100% A; and 40–45 min, 100–20% A. The compound with RT 33.5 min was assumed to be 20NP 20α-H isomer, collected and used for microbiological tests.

2.2.2. 3-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-cholestane (3NC) synthesis

5α-cholestan-3-one (100 mg, 0.260 mmol) and ammonium acetate (405 mg, 5.20 mmol) were mixed with 10 ml of dry argon-saturated methanol and then kept at 40 °C for 1 h, followed by $NaBH_3CN$ (330 mg, 5.20 mmol) addition (6 equal portions within 60 min). The solution was stirred 24 h until full conversion of the substrate took place. Then, 5 ml of cold 0.5 M hydrochloric acid was mixed with the solution. One hour later, 5 ml of cold 0.5 M sodium hydrocarbonate was added. Subsequently, the mixture was extracted with *n*-hexane (4 × 10 ml). The combined extract was washed with 10 ml of cold water and evaporated to dryness. The substance was mixed with 5 ml of a methanol:chloroform (1:3, v:v), NBD-Cl (100 mg, 0.50 mmol) in 1 ml methanol and sodium carbonate (0.5 g) saturated water solution. The mixture was stirred in the dark overnight at room temperature. Then, 10 ml of cold 1 N hydrochloric acid and 40 ml chloroform were added; the organic phase was concentrated and then the fluorescent products were purified using silica gel-based column chromatography with a benzene:ethyl acetate gradient as the mobile phase. Dark orange solid was obtained with 15% yield.

^{1}H NMR: (300 MHz, $CDCl_3$) δ = 8.16 ppm (d, 1H, J = 7.5 Hz, benzoxadiazole C6-**H**), 7.13 (d, 1H, J = 7.5, benzoxadiazole C5-**H**), 2.57 (m, 1H, C3-**H**), 1.04 (s, 6H, C18-**H** & C19-**H**), 0.91–0.96 (m, 9H, C21-H, C26-H & C27). **ESI-MS**: *m/z* 549 $[M\text{-}H]^-$, **EI-MS**: *m/z* 550 (M^+, 43%), 533, 410, 371, 355, 215. **UV–Vis**: λ_{max} = 470 nm (MeOH). **Fluorimetry**: λ_{max} (emission) = 560 nm (MeOH).

Fluorescent 3NC, possessing highest hydrophobicity among fluorescent compounds obtained, was purified by HPLC as described above using 40:60 (v:v) acetonitrile:methanol (solution (A) and water (B) gradient was used according to the following protocol: 0–5 min, 5% A; 5–30 min, 5–100% A; 30–60 min, 100% A; and 60–70 min, 100–5% A). The compound with RT 53.7 min was assumed to be 5α-H,3β-(NBD-amino)- (or 3α-H) isomer [18,19], collected and used for microbiological tests.