Biochemical Pharmacology 98 (2015) 167–181

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

# Chemical inhibition of fatty acid absorption and cellular uptake limits lipotoxic cell death

Constance Ahowesso[a], Paul N. Black[a], Nipun Saini[a], David Montefusco[a], Jessica Chekal[a], Chrysa Malosh[b,c], Craig W. Lindsley[b,c,d], Shaun R. Stauffer[b,c,d], Concetta C. DiRusso[a,*]

[a] Department of Biochemistry, University of Nebraska–Lincoln, Lincoln, NE 68588-0664, United States
[b] Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232, United States
[c] Vanderbilt Specialized Chemistry Center for Probe Development (MLPCN), Nashville, TN 37232, United States
[d] Department of Chemistry, Vanderbilt University, Nashville, TN 37232, United States

ARTICLE INFO

*Article history:*
Received 22 July 2015
Accepted 3 September 2015
Available online 21 September 2015

*Chemical compounds studied in this article:*
5′-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3′-indoline]-2′-one

*Keywords:*
FATP2 inhibitor
Lipotoxicity
Lipid droplet
Fatty acid transport

ABSTRACT

Chronic elevation of plasma free fatty acid (FFA) levels is commonly associated with obesity, type 2 diabetes, cardiovascular disease and some cancers. Experimental evidence indicates FFA and their metabolites contribute to disease development through lipotoxicity. Previously, we identified a specific fatty acid transport inhibitor CB16.2, a.k.a. Lipofermata, using high throughput screening methods. In this study, efficacy of transport inhibition was measured in four cell lines that are models for myocytes (mmC2C12), pancreatic β-cells (rnINS-1E), intestinal epithelial cells (hsCaco-2), and hepatocytes (hsHepG2), as well as primary human adipocytes. The compound was effective in inhibiting uptake with $IC_{50}$s between 3 and 6 μM for all cell lines except human adipocytes (39 μM). Inhibition was specific for long and very long chain fatty acids but had no effect on medium chain fatty acids (C6–C10), which are transported by passive diffusion. Derivatives of Lipofermata were evaluated to understand structural contributions to activity. Lipofermata prevented palmitate-mediated oxidative stress, induction of BiP and CHOP, and cell death in a dose-dependent manner in hsHepG2 and rnINS-1E cells, suggesting it will prevent induction of fatty acid-mediated cell death pathways and lipotoxic disease by channeling excess fatty acids to adipose tissue and away from liver and pancreas. Importantly, mice dosed orally with Lipofermata were not able to absorb $^{13}$C-oleate demonstrating utility as an inhibitor of fatty acid absorption from the gut.



## 1. Introduction

Long-chain fatty acids (LCFAs) are vital dietary components and contribute to normal metabolic homeostasis including, for example, energy generation and storage, plasma membrane synthesis, and protein anchoring. Excess free fatty acids (FFA) are esterified and stored as triglycerides in lipid droplets in various cell types. These subcellular compartments are dynamic and stored fatty acids can be mobilized by the actions of cellular lipases in a process regulated by hormones and by proteins associated with the droplets. Apart from adipocytes, most cells have a limited capacity for lipid storage and when this capacity is exceeded, cell death may result from a process called lipotoxicity [1–3]. Current evidence indicates free fatty acids and/or their metabolites mediate cell death [2,4,5]. Chronic imbalances in lipid flux and metabolism often cause a variety of metabolic abnormalities and pathologies,

*Abbreviations:* Acsl, long chain acyl CoA synthetase; BiP, binding immunoglobulin protein; BSA, bovine serum albumin; $C_1$-BODIPY-$C_{12}$, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; CHOP, CCAAT/-enhancer-binding protein homologous protein; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ELSD, evaporative light scattering detection; FA, fatty acid; FFA, free fatty acid; FATP2, fatty acid transport protein 2; GC/MS, gas chromatography/mass spectrometry; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HPLC, high performance liquid chromatography; LCFA, long chain fatty acid; LC/MS, liquid chromatography/mass spectrometry; Lipofermata, CB16.2, 5′-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3′-indoline]-2′-one; MEM, minimum essential media; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NR, Nile Red; PA, palmitic acid; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RFU, relative fluorescence units; RP-HPLC, reverse phase high performance liquid chromatography; SFA, saturated fatty acid; T2D, type II diabetes mellitus; TFA, trifluoroacetic acid; UFA, unsaturated fatty acid; VLCFA, very long chain fatty acid.

* Corresponding author at: Department of Biochemistry, N241 Beadle Center, University of Nebraska–Lincoln, NE 68588-0664, United States. Fax: +1 4024727842.
*E-mail address:* cdirusso2@unl.edu (C.C. DiRusso).

http://dx.doi.org/10.1016/j.bcp.2015.09.004
0006-2952/

including hyperlipidemia, type 2 diabetes mellitus (T2D), nonalcoholic fatty liver disease (NAFLD), heart disease, and some cancers [6–8]. Obesity is a common initiating condition for these diseases due to chronic hyperlipidemia and elevated free fatty acids. Lipid overload in pancreatic β-cells may lead to dysregulated insulin secretion and apoptotic cell death, both of which may contribute to the genesis of the diabetic state [9,10]. Lipoapoptosis is also observed in the heart and contributes to the development of heart failure [11–13]. In liver, chronically elevated fat results in a condition called non-alcoholic fatty liver disease (NAFLD) that can lead to steatohepatitis (NASH) and eventually to non-reversible cirrhosis [14,15]. Therefore, there is much interest in preventing lipotoxicity that leads to metabolic dysfunction and disease.

The uptake of unesterified LCFAs into mammalian cells may occur either through passive diffusion or protein-mediated mechanisms [16–18]. Passive diffusion is operable in all cell types and is limited by the membrane composition, the ability of the fatty acid to flip from one side of the membrane to the other, and the presence of intracellular proteins that bind and extract the fatty acid from the membrane (e.g., FABP or acyl-CoA synthetase (Acs)). Cells and organs that are specialized in lipid metabolic processes also express proteins involved in transport of long- and very-long chain fatty acids, which are diffusion limited. Most information on protein-mediated fatty acid transport has been acquired for adipose tissue [19,20], intestine [21], liver [22,23], and heart [24,25]. Specific membrane proteins that increase the uptake of LCFAs when overexpressed in cultured mammalian cells have been identified. The most prominent and best characterized of these are fatty acid translocase (FAT)/CD36 [22], the fatty acid transport proteins (FATPs) [26,27] and long-chain fatty acyl-coenzyme A synthetase (Acsl) [16,28,29].

The FATPs are bifunctional proteins, which transport long chain fatty acids (LCFA) into cells and activate very long chain fatty acids (VLCFA) by esterification with coenzyme A [30]. The recent characterizations of FATP deletion (KO) and transgenic mouse strains, as well as data gained from human subjects have clearly demonstrated that FATPs are important determinants of lipid distribution among different organs and can dynamically change LCFA uptake in response to altered nutrient availability [24,31–33]. Recently, several FATP-specific fatty acid transport inhibitors were selected in a high throughput screen against human FATP2b, a splice variant that is deficient in VLCFA activation but that retains LCFA transport activity [34]. Among the hits, CB16.2 (5′-bromo-5-phenyl-spiro[3H-1,3,4-thiadiazole-2,3′-indoline]-2′-one) now called Lipofermata was identified as a compound that specifically blocked human FATP2-mediated fatty acid uptake without impacting other cellular functions [34]. Lipofermata inhibits fatty acid uptake into HepG2 and Caco-2 cells (models for human hepatocytes and enterocytes, respectively) with high specificity and affinity [34]. Both cell lines express FATP2. In contrast, inhibition of fatty acid uptake by Lipofermata into murine 3T3L1 adipocytes, which do not express FATP2 to a significant extent, is at least 10-times less effective. This suggests Lipofermata may be a tractable tool to determine the underlying mechanistic features of FATP-dependent transport to specifically differentiate between effects on different cell types and organ systems susceptible to lipotoxicity.

In the present study, we assessed the potency of Lipofermata to inhibit fatty acid transport across the intestine in mice and in cell lines that are models for pancreatic β cells and myocytes, as well as primary human adipocytes. The compound acts as a non-competitive inhibitor specific for long and very long chain fatty acids. However, it is ineffective in inhibiting the uptake of medium chain fatty acids (C6–C10). In mice, the compound inhibited absorption of oleate labeled with $^{13}C$. Importantly, we demonstrate that Lipofermata specifically prevents cellular dysfunction and death caused by exposure to saturated fatty acids.

## 2. Materials and methods

### 2.1. Chemicals, reagents, and analytical methods

Lipofermata/CB16.2 (5-bromo-5′-phenyl-3′H-spiro [indole-3,2′-[1,3,4] thiadiazol]-2(1H)-one) and structurally related compounds were either purchased from ChemBridge Corporation (San Diego, CA, USA) or was synthesized by the Vanderbilt Specialty Chemistry Center (Nashville, TN, USA) according to the scheme in Fig. 1. $C_1$-BODIPY-$C_{12}$ (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid), BODIPY-FL-C5 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid) and BODIPY-FL-C16 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-hexadecanoic acid) were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA). Tyloxapol and $^{13}C$-oleate (uniformly labeled) were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA).

For analytical characterization of synthesized compounds, low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer. Analytical thin layer chromatography was performed on Analtech silica gel GF 250 micron plates (Spectrum Chemical Mfg. Corp., New Brunswick, NJ). Analytical HPLC was performed on an Agilent HP1100 (Agilent Technologies, Inc., Santa Clara, CA, USA) with both UV detection at 214 and 254 nm and ELSD followed by LC/MS (J-Sphere80-C18, 3.0 × 50 mm using a 4.1 min gradient of 5%[0.05%TFA/acetonitrile]:95%[0.05% TFA/$H_2O$] to 100%[0.05%TFA/acetonitrile]. Preparative RP-HPLC purification was performed on a Gilson preparative UV-based

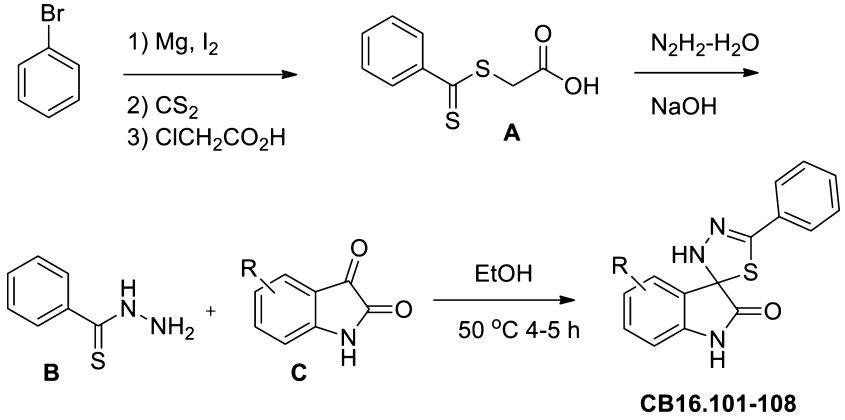


**Fig. 1.** Synthesis scheme for Lipofermata and structural derivatives. For details, see Section 2.2.