Life Sciences 135 (2015) 35–42

Contents lists available at ScienceDirect

Life Sciences

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

Cineromycin B isolated from *Streptomyces cinerochromogenes* inhibits adipocyte differentiation of 3T3-L1 cells via Krüppel-like factors 2 and 3

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ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 25 May 2015
Accepted 27 May 2015
Available online 17 June 2015

Keywords:
3T3-L1 differentiation
Cineromycin B
GATA binding protein
Krüppel-like factor

ABSTRACT

Aims: 3T3-L1 cells are preadipocytes and often used as a model for cellular differentiation to adipocytes; however, the mechanism of this differentiation is not completely understood even in these model cells. In this study, we sought to identify a unique anti-adipogenesis agent from microorganisms and to examine its mechanism of action to gain knowledge and create a tool and/or seed compound for anti-obesity drug discovery research.
Main method: Screening for anti-adipogenesis agents from microorganisms was performed using a 3T3-L1 cell differentiation system, and an active compound was isolated. The inhibitory mechanism of the compound was investigated by measuring the expression of key regulators using quantitative real-time PCR and Western blot analysis.
Key findings: The compound with anti-adipogenic activity in 3T3-L1 cells was identified as cineromycin B. Cineromycin B at 50 μg/mL suppressed intracellular lipid accumulation and the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα), which are master regulators of adipocyte differentiation. Further investigations showed that cineromycin B increased significantly the mRNA expression of two negative regulators of adipocyte differentiation, Krüppel-like factor (KLF) 2 and KLF3, at an early stage of the differentiation. The results of siRNA transfection experiments indicated that cineromycin B is a unique adipocyte differentiation inhibitor, acting mainly via upregulation of KLF2 and KLF3, and these KLFs may play a role in the early stage of differentiation.
Significance: Cineromycin B inhibited adipocyte differentiation in 3T3-L1 cells mainly via upregulation of KLF2 and KLF3 mRNA expression at an early stage of the differentiation.

1. Introduction

Obesity is considered to be a causal factor of various diseases, such as cardiovascular diseases, diabetes mellitus type 2, obstructive sleep apnea, certain types of cancer, and osteoarthritis [1]. Expanded adipose tissue shows an increased number and size of adipocytes due to enhancement of the differentiation to adipocytes from preadipocytes and lipid accumulation [2].

3T3-L1 cells are commonly used as preadipocytes in studies of adipocyte differentiation. These cells directly differentiate to adipocytes upon addition of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), and insulin [3]. Studies of the molecular mechanism underlying adipocyte differentiation have indicated that many genes and proteins play important roles during the formation of fat cells [4]. For convenience, a hypothetical schematic illustration of adipocyte differentiation is set out in Fig. 1. In the process of differentiation, upstream regulators, CCAAT/enhancer binding protein-beta (C/EBPβ) and C/EBPδ, regulate the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and C/EBPα [5]. PPARγ and C/EBPα are necessary for adipocyte differentiation, and positively cross-regulate each other [6]. In particular, PPARγ plays a central role in adipocyte differentiation because preadipocytes do not differentiate to adipocytes without PPARγ expression [7,8].

Recently, some regulatory factors associated with the early stages of adipocyte differentiation have been identified, including GATA binding protein (GATA) family and Krüppel-like factor (KLF) family members. GATA-2 and GATA-3 were reported to be anti-adipogenic factors that bind directly to the GATA site in the PPARγ promoter [9]. Members of the KLF family have a characteristic zinc-finger motif in the C terminus, and some of the 17 family members have been reported to play roles in cell proliferation, growth, and differentiation in mammals [10,11]. Among these family members, KLF2, 3, 4, 5, 6, 7, and 15 are considered to be associated with adipocyte differentiation. KLF2, 3, and 7 are highly

Abbreviations: ANOVA, analysis of variance; $CaCO_3$, calcium carbonate; $CDCl_3$, chloroform-*d*; $CHCl_3$, chloroform; CH_3CN, acetonitrile; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; $FeSO_4$, iron (II) sulfate; HPLC, high-performance liquid chromatography; K_2HPO_4, potassium phosphate dibasic; LC-ESI-MS, liquid chromatography electro spray ionization mass spectrometry; MeOH, methanol; $MgSO_4$, magnesium sulfate; NaCl, sodium chloride; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PDA, photodiode array.

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http://dx.doi.org/10.1016/j.lfs.2015.05.020
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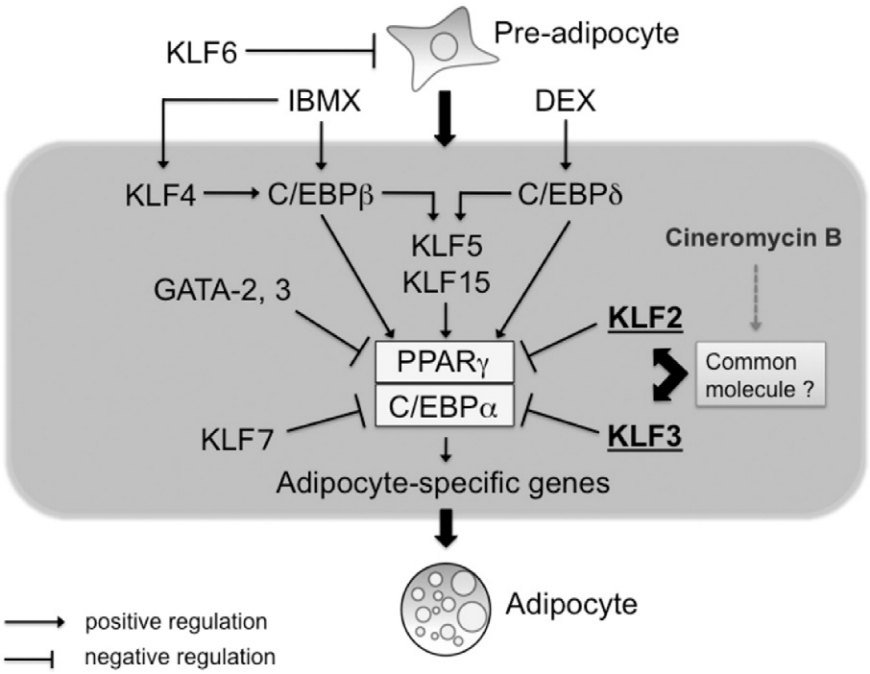

Fig. 1. A hypothetical schematic illustration of adipocyte differentiation in 3T3-L1 cells.

expressed in preadipocytes and their expression levels are reduced after induction. Because overexpression of KLF2, 3, or 7 in 3T3-L1 cells inhibits adipocyte differentiation, these factors apparently act as negative regulators of the differentiation [12,13,14]. In contrast, expression levels of KLF4, 5, 6, and 15 were reported to increase after induction. Because suppression of KLF4, 5, 6, or 15 inhibits adipocyte differentiation, these factors apparently act as positive regulators of the differentiation [15,16,17,18]. The role of individual KLFs in the differentiation has been reported, as mentioned above; however, relationships between actions of KLF family members in the differentiation are unclear.

In the course of screening for anti-adipogenic agents from microorganisms, we identified cineromycin B as an active compound. We found that cineromycin B regulated expression levels of several KLF family members at the early stages of the differentiation. Further studies on the mechanism of action with siRNA transfection experiments revealed that cineromycin B inhibited the differentiation mainly via KLF2 and KLF3. We describe here the isolation and identification of cineromycin B and its mechanism of action as an inhibitor of adipocyte differentiation.

2. Material and methods

2.1. General experimental procedures

NMR spectra were measured using a JMN-ECA-600 NMR spectrometer (Jeol, Tokyo, Japan) using tetramethylsilane as an internal standard in $CDCl_3$. HPLC chromatograms and UV–Vis spectra were recorded using a GL-7410 HPLC pump and GL-7452 PDA detector (GL Science, Tokyo, Japan) with a CH_3CN–H_2O solvent system. HPLC fractionation used an L-6200 HPLC pump and L-4200 UV detector (Hitachi, Ibaraki, Japan). LC-ESI-MS data were measured using a 1200 series HPLC system and an API-3200 triple quadrupole mass spectrometer (Applied Biosystems, Tokyo, Japan). Optical density (OD) was measured with a SH-1000 Lab microplate reader (Corona, Ibaraki, Japan). Cosmosil 5C18-AR-II (Nacalai Tesque, Kyoto, Japan) was used for chromatography. Quantitative real-time polymerase chain reaction (qPCR) was performed using Chromo4 (Bio-Rad Laboratories Inc., Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Culture and differentiation of 3T3-L1 cells

3T3-L1 preadipocytes (Human Science, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) until confluence. Two days after reaching confluence, cells were stimulated to differentiate with differentiation medium (day 0) containing DMEM with 10% FBS, 0.5 mM IBMX, 1.7 μM insulin, and 0.25 μM DEX for 3 days. Then, differentiation medium was exchanged for maintenance medium containing DMEM supplemented with 10% FBS and 1.7 μM insulin, and incubation was continued for 2 days (day 3), followed by 2 days of incubation with maintenance medium (day 5). All media contained 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The test samples were dissolved in dimethyl sulfoxide (DMSO), and DMSO was used as a control. Our preliminary study showed that 0.3% DMSO in media did not affect cell viability or differentiation. The cultures were treated with test samples for the whole culture period (days 0 7).

2.3. Measurement of lipid level by Oil Red O staining

Lipid droplets in cells were stained with Oil Red O (Sigma-Aldrich, Tokyo, Japan) at the end of the culture period (day 7). The cells were washed three times with phosphate-buffered saline (PBS) and fixed with 10% formalin at room temperature for 20 min. After fixation, cells were washed once with PBS and stained with freshly diluted Oil Red O solution (three parts 0.6% Oil Red O in isopropyl alcohol and two parts water) for 15 min. Cells were then washed twice with PBS and visualized. For quantitative analysis, Oil Red O staining was dissolved with 4% NP-40 in isopropyl alcohol and the optical density at 520 nm (OD_{520}) was measured using a microplate reader. Lipid accumulation in cells treated with each sample was expressed as the percentage ratio of the optical density of the tested cells to the control cells treated with DMSO alone.

2.4. Cineromycin B

We performed screening for anti-lipid accumulation activity using the 3T3-L1 differentiation method from our library of ~1500 microorganism