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Pharmacological characterization of a series of aryl-sulfonamide derivatives that potently and selectively inhibit monoacylglycerol acyltransferase 2

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ABSTRACT

Monoacylglycerol acyltransferase (MGAT) 2 is an endoplasmic reticulum membrane enzyme that catalyzes the synthesis of diacylglycerol (DAG) from fatty acyl-CoA and monoacylglycerol as substrates. It is important for the resynthesis of triacylglycerol in the intestine. We have identified a series of aryl-sulfonamide MGAT2 inhibitors and demonstrated pharmacological inhibition of MGAT2 improved hyperlipidemia, obesity, and diabetes in animal models. However, its mechanism of action has not been elucidated in molecular and cellular levels. In the present study, we have characterized a series of aryl-sulfonamide derivatives that potently and selectively inhibit human MGAT2 and determined their pharmacological profiles. Analyses on the molecular mechanism of a representative aryl-sulfonamide MGAT2 inhibitor revealed a reversible inhibitory activity and a binding activity to MGAT2. The aryl-sulfonamide derivatives exhibited potent inhibitory activities against both human and mouse intestinal MGAT activities, which were correlated to those determined using recombinant human and mouse MGAT enzymes. We have developed a cellular assay using Liquid Chromatography-Mass Spectrometry and confirmed that the aryl-sulfonamide derivatives suppressed DAG synthesis in the cellular context. We have thus elucidated their pharmacological profiles and provided the fundamental clues for understanding the molecular and cellular actions of the aryl-sulfonamide MGAT2 inhibitors.

1. Introduction

Monoacylglycerol acyltransferase (MGAT) 2 (EC 2.3.1.22) is a membrane acyltransferase that synthesizes diacylglycerol (DAG) using fatty acyl-CoA as a donor of an acyl moiety and monoacylglycerol (MAG) as an acceptor (Scheme 1) (Cao et al., 2003; Lockwood et al., 2003; Yen and Farese Jr., 2003). MGAT2 is involved in the resynthesis of triacylglycerol (TAG) *via* the MGAT pathway in enterocytes. MGAT2 is thus involved in the metabolism of TAG in the intestine (Cao et al., 2012) and is crucial for dietary fat absorption from the intestinal lumen. It was reported that MGAT2 deficiency protected mice from high-fat diet-induced obesity and insulin resistance with enhanced energy expenditure (Nelson et al., 2011; Tsuchida et al., 2012; Yen et al., 2009). Therefore, it was suggested that the inhibition of MGAT2 ameliorates obesity and metabolic diseases.

Small-molecule MGAT2 inhibitors have been reported by multiple groups including us (Barlind et al., 2013; Okuma et al., 2015; Sato et al., 2015a, 2015b). MGAT2 belongs to the membrane acyl transferase family and the enzymatic characterization has been reported by conventional methods (Cao et al., 2003; Lockwood et al., 2003; Yen and Farese Jr., 2003). Although lack of a robust assay system has hindered the pharmacological analyses, the evaluation methods for MGAT2 inhibitors in cellular and biochemical assays have been developed with synthetic MGAT2 inhibitors (Ma et al., 2016; Onorato et al., 2015; Seethala et al., 2008). In the recent studies, we performed a tandem mass spectrometry (MS/MS)-based high-throughput screening (HTS) to identify intestinal MGAT inhibitors and have initiated a compound optimization program (Sato et al., 2015a). We demonstrated that an aryl-sulfonamide MGAT2 inhibitor improved dyslipidemia, obesity, and diabetes in animal models (Take et al., 2016). However, the pharmacological mode of action has not been elucidated for this series of MGAT2 inhibitors at molecular and cellular levels. Also, the inhibitory activities against human and mouse intestinal MGAT activities have not been clarified.

In the present study, we used kinetic and binding experiments to elucidate the mode of action of an aryl-sulfonamide MGAT2

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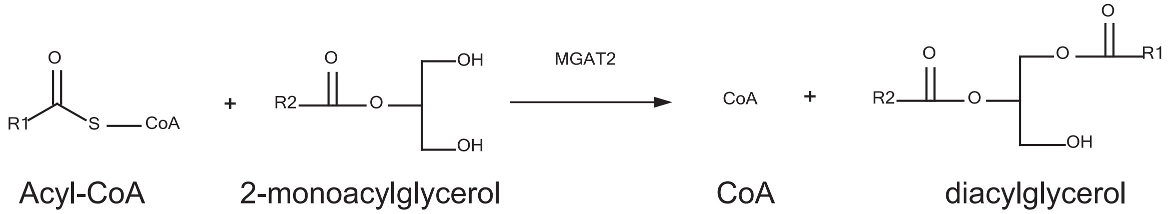

Scheme 1. Enzymatic reaction catalyzed by MGAT2 with R1 as an acyl donor and R2 as an acyl moiety in 2-monoacylglycerol.

inhibitor. In addition, microsomal and cellular MGAT assays were applied to demonstrate the effectiveness of the series of aryl-sulfonamide derivatives. Our characterization support understanding the pharmacological profile of the aryl-sulfonamide derivatives that potently and selectively inhibit MGAT2 activity.

2. Materials and methods

2.1. Materials

Compound B, compound C, and compound J were synthesized as reported previously (Sato et al., 2015b). Compound H was synthesized as reported previously (Sato et al., 2015a). Compound D, compound E, compound F, compound G, compound I, compound K, and compound L were synthesized in house. The substrates 2-oleoyl-glycerol and $^{13}C_{18}$-oleoyl-CoA and an internal standard 1,2 dioleoyl-*sn*-glycerol were purchased from Sigma-Aldrich (St. Louis, MO). Mouse and mixed human intestinal microsomes were purchased from XENOTECH (Lenexa, KS). $^{13}C_{18}$-oleic acid (Sigma-Aldrich) complexed with bovine serum albumin (BSA) (Sigma-Aldrich) was prepared as reported (Goldstein et al., 1983).

2.2. Protein preparation

The full-length coding sequences of human diacylglycerol acyltransferase 1 (DGAT1) and diacylglycerol acyltransferase 2 (DGAT2) are identical to NCBI accession numbers AB057815 and NM_032564, respectively. These genes were cloned by PCR amplification. The resulting PCR products were cloned into the pFastBac vector for expression in *Spodoptera frugiperda* 21 (Sf-21) insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA). A FLAG tag was attached to the N-terminus of all genes of interest. The pFastBac vector containing a cDNA fragment of the relevant gene was transformed into DH10Bac *Escherichia coli* cells to generate a recombinant bacmid. High-titer recombinant baculovirus was generated by transfecting the bacmid DNA into insect cell lines, followed by several rounds of amplification to increase the viral titer. After infection with recombinant baculoviruses for an optimized time, insect cells were harvested and homogenized in ice-cold BufferA (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4/K_2HPO_4 at pH 7.4) and pelleted by centrifugation at 500 rpm for 10 min. Following this, a crude mitochondrial pellet was obtained by centrifugation of the pooled supernatants at 40,000 rpm for 60 min. The pellet was resuspended in BufferA and harvested at 10,000 rpm for 10 s two times. The full-length coding sequences of human MGAT2, mouse MGAT2, and human MGAT3 are identical to NCBI accession numbers NM_025098, NM_177448, and NM_001109436, respectively. The sequences were subcloned into the pcDNA3.3 vector (Life Technologies, Carlsbad, CA) with a FLAG tag attached to the N-terminal region to generate the expression plasmids in mammalian cells. To prepare overexpressed membranes, the expression vector was transiently transfected into FreeStyle293 cells (Life Technologies) using NeoFection (ASTEC, Fukuoka, Japan) for human MGAT2 and mouse MGAT2 and into COS-7 cells for human MGAT3 according to the instruction manual. After culture for 2 days, cells were collected and homogenized in ice-cold 20 mM Tris–HCl buffer (pH 7.5 at 25 °C) containing 1 mM EDTA and 300 mM sucrose. Cell homogenates were centrifuged (2000 rpm, 10 min, 4 °C), and the supernatant was recovered. Total membrane fractions were isolated by ultracentrifugation (35,000 rpm, 60 min, 4 °C). Pellets were resuspended in the same buffer and stored at −80 °C. The protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology) according to the instruction manual.

2.3. Liquid Chromatography-Mass Spectrometry (LC/MS)-based enzymatic assay

The reaction was run in 20 µl of an assay buffer [100 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 200 mM sucrose, 0.01% Tween 20, 2 mM DTT, 0.01% bovine serum albumin (BSA), and 5% DMSO] supplemented with 5 µM $^{13}C_{18}$-oleoyl-CoA (Sigma-Aldrich, St. Louis, MO), 5 µM 2-oleoyl-glycerol (Sigma-Aldrich), and 0.3 µg/ml MGAT2, using a 384-well assay plate (REMP #23490-320). After incubation at room temperature for 30 min, the reaction was stopped with 60 µl of acetonitrile containing 0.88% formic acid, and 1.3 µM 1,2-dioleoyl-glycerol (Sigma-Aldrich) was added as an internal standard. The samples were mixed and centrifuged to precipitate the proteins. High-throughput online solid-phase extraction was performed using the RapidFire 300 System (Agilent Technologies, Santa Clara, CA). The samples were loaded onto an SPE C4 cartridge (Agilent Technologies) using 10 mM ammonium formate and 0.2% formic acid in acetonitrile/deionized water (30/70, v/v) at a flow rate of 1.5 ml/min and eluted using 5 mM ammonium formate and 0.2% formic acid in acetonitrile/deionized water (98/2, v/v) at a flow rate of 1.25 ml/min. The injection needle was washed with deionized water followed by acetonitrile. The aspiration time (injection volume 10 µl), load/wash time, elution time, and re-equilibration time were adjusted to 500, 2500, 5000, and 1000 ms, respectively, for a total cycle time of approximately 10 s

Mass spectrometric analysis of analyte formation was performed using an API-4000 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA) equipped with an electro-spray ion source (TurboIon Spray) operated in the positive selected reaction monitoring (SRM) mode. The SRM transitions for $^{13}C_{18}$-dioleoylglycerol as a reaction product and dioleoylglycerol as an internal standard were set as 656.6/339.2 and 638.5/339.2, respectively. The SRM conditions for both species were set as follows: declustering potential (DP), 71 V; collision energy (CE), 31 V; and collision exit potential (CXP), 18 V. Parameters were optimized as follows: capillary temperature, 50 °C and ion spray voltage, 5.5 kV. Collision gas, curtain gas, ion source gas 1, and ion source gas 2 pressures were set at 6, 10, 60, and 40 psi, respectively. Analytical data were acquired and analyzed using Analyst software (version 1.5.0; AB SCIEX).

The inhibitory activity was calculated as follows: % inhibition $=(A-B)/(A-C)\times 100$, where A, B, and C are the peak area ratio