Discovery of a Novel Class of Diacylglycerol Acyltransferase 2 Inhibitors with a 1*H*-Pyrrolo[2,3-*b*]pyridine Core

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Diacylglycerol acyltransferase 2 (DGAT2), which catalyzes the final step in triacylglycerol (TG) synthesis, is a key enzyme associated with hepatic steatosis and insulin resistance. Here, using an *in vitro* screen of 20000 molecules, we identified a class of compounds with a substituted 1*H*-pyrrolo[2,3-*b*]pyridine core which proved to be potent and selective inhibitors of human DGAT2. Of these compounds, H2-003 and -005 exhibited a considerable reduction in TG biosynthesis in HepG2 hepatic cells and 3T3-L1 preadipose cells. These compounds exert DGAT2-specific-inhibitory activity, which was further confirmed in DGAT2- or DGAT1-overexpressing HEK293 cells. In addition, these compounds almost completely abolished lipid droplet formation in 3T3-L1 cells when co-treated with a DGAT1 inhibitor, which was not attained using either a DGAT2 or DGAT1 inhibitor alone. Collectively, we identified two DGAT2 inhibitors, H2-003 and -005. These compounds will aid in DGAT2-related lipid metabolism research as well as in therapeutic development for the treatment of metabolic diseases associated with excessive TG.

Key words metabolic disease; triacylglycerol; diacylglycerol acyltransferase 2 (DGAT2); small molecule inhibitor

Triacylglycerols (TGs), a major type of neutral lipid, are essential forms of energy storage composed of three fatty acids attached to a glycerol in eukaryotes.¹⁾ However, positive energy balance results in excessive accumulation of TG throughout the body and leads to several metabolic diseases, such as obesity, hyperlipidemia, hypertension, hepatic steatosis, and insulin resistance.^{2–4)} Therefore, inhibiting TG biosynthesis has been suggested as one of therapeutic strategies for treating these diseases.

Diacylglycerol acyltransferase (DGAT) enzymes, which exist as two isoforms DGAT1 and DGAT2, catalyze the final step in TG biosynthesis. These isozymes are encoded by two distinct genes and share no amino acid sequence homology.⁵⁾ Furthermore, DGAT1 is highly expressed in the small intestine, whereas DGAT2 is primarily expressed in the liver.⁶⁾ This differential expression pattern suggests a fundamentally different role for each DGAT enzyme in TG metabolism.

Several lines of evidence have shown that DGAT2 is more essential than DGAT1 for homeostatic control of TGs *in vivo*. DGAT2 knockout mice are severely deficient in TGs (approximately 90% TG reduction) and have impaired skin barrier function, leading to early death.⁷⁾ In contrast, DGAT1 knockout mice are still viable with a moderate reduction in TG (approximately 50% TG reduction).^{8,9)} In addition, inhibition of DGAT2 expression by antisense oligonucleotides reduced hepatic TG content and increased fatty acid oxidation, thereby reversing diet-induced hepatic steatosis and insulin resistance in rodents.^{10,11)} Conversely, upregulation of DGAT2 expression increased cytoplasmic TG content and lipid droplets (LDs) in rat hepatocytes¹²⁾ and adipocytes.¹³⁾ Similarly, liver-directed DGAT2 overexpression caused hepatic TG accumulation¹²⁾

and promoted hepatic insulin resistance¹⁴⁾ in mice. Therefore, inhibition of DGAT2 activity, particularly by a small molecule, is expected to be a feasible therapeutic strategy for treating hepatic steatosis and its complications, such as insulin resistance.

Several DGAT2-selective inhibitors have been reported recently, such as JNJ-DGAT2-A,¹⁵⁾ JNJ-DGAT2-B,¹⁵⁾ indolyl acrylamide analogues,¹⁶⁾ and compound 122.¹⁷⁾ However, the pharmacological validation of their use, particularly in animal models, remains to be examined. Still, DGAT2 inhibitors with different modes of action or pharmacophores will be useful to advance our understanding of DGAT2 biology and develop therapeutic drugs for treating several metabolic diseases.

In this study, we sought to discover another class of potent and selective DGAT2 inhibitors and validate their pharmacological effect at the cellular level. A high-throughput screen using a robust *in vitro* DGAT2 assay identified a novel class of compounds with a 1*H*-pyrrolo[2,3-*b*]pyridine core as DGAT2 inhibitors. Various cell-based assays clearly confirmed the selective effects of H2-003 and -005 on DGAT2, leading to a considerable suppression of TG accumulation in several mammalian cells.

MATERIALS AND METHODS

Materials 3T3-L1 and HepG2 cells were purchased from ATCC, and HEK293 Tet-on cells were obtained from Clontech. Fatty acid-free BSA and *sn*-1,2-dioleoylglycerol were obtained from Sigma-Aldrich. [³H]-Oleoyl CoA, [¹⁴C]-oleoyl CoA, and [¹⁴C]-glycerol were purchased from PerkinElmer, Inc. The Bac-to-Bac Baculovirus Expression System was purchased from GIBCO. An antibody against DGAT2 was purchased from Abcam. Reference DGAT1-selective inhibitor (*N*-(6-morpholinopyridin-3-yl)-1-phenyl-

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3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide) was domestically synthesized.

Cell Culture 3T3-L1 fibroblasts were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). HepG2 and HEK293 Tet-on cells were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Tet System Approved Serum, Clontech), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin.

Protein Expression and Membrane Preparations for *in Vitro* Enzymatic Assay Total membrane containing overexpressed human DGAT1 or DGAT2 was prepared as described previously.¹⁷ Briefly, human DGAT1 and DGAT2 were overexpressed in Sf-9 insect cells using the Bac-to-Bac Baculovirus Expression System according to the manufacturer's instructions. The DGAT1 and DGAT2 cDNA clones were amplified by polymerase chain reaction (PCR) and inserted into the pFastBac1 donor vector to obtain recombinant baculoviruses. Sf-9 cells infected with the resultant baculovirus at 10 MOI for 72 h were harvested and homogenized in sucrose solution (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT)). The homogenates were centrifuged at 600 $\times g$ for 15 min, and the resulting supernatants were further centrifuged at 100000 $\times g$ for 1 h. The collected membranes were resuspended, and the protein concentration was measured using the Bradford protein assay method.

High-Throughput Screening of Human DGAT2 Inhibitors The high-throughput screening to discover small-molecule inhibitors of DGAT2 was achieved using basic FlashPlate (PerkinElmer, Inc.), which is based on the scintillation proximity assay. DGAT2 activity was assayed in a solution containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 5 mM MgCl₂, 1 mg/mL bovine serum albumin (BSA), 100 μ M diacylglycerol, 10 μ M [³H]-oleoyl CoA, 8 μ g DGAT2-containing membrane, and 10 μ M compound in a final reaction volume of 100 μ L in a 96-well format Basic FlashPlate. The reaction mixture was incubated for 2 h at 37°C, and the enzymatic reaction was stopped by adding 100 μ L of isopropanol. The plates were sealed and incubated overnight at room temperature. The newly synthesized [³H]-labeled TG was in close proximity to the coated scintillant in the FlashPlate during the incubation. We quantified the plates by scintillation counting with a Micro-beta (PerkinElmer, Inc.) plate reader.

Determination of *in Vitro* DGAT Enzymatic Activity DGAT activity assays were carried out as described previously.¹⁷ Briefly, DGAT activity in total membranes prepared from DGAT2- or DGAT1-overexpressing Sf-9 and HEK293 Tet-on cells was determined by measuring the formation of [¹⁴C]-triacylglycerol from [¹⁴C]-oleoyl CoA. The reaction mixture for the DGAT2 assay contained 175 mM Tris (pH 7.5), 5 mM MgCl₂ (100 mM MgCl₂ for DGAT1 assay), 200 μ M *sn*-1,2-diacylglycerol, 20 μ M [1-¹⁴C]-oleoyl CoA (5.5 μ Ci), 2 mg/mL BSA, and 32 μ g of the membrane protein. The mixture was incubated for 20 min at 37°C and then the reaction was stopped by the addition of 1.5 mL of stop solution [2-propanol–heptane–water (80:20:2, v/v/v)] and vortexed with 1.0 mL of heptane and 0.5 mL of water. The top heptane phase was collected and washed with 2.0 mL alkaline ethanol solution [ethanol–0.5 N NaOH–water (50:10:40, v/v/v)]. The radioac-

tivity of the top phase was determined by liquid scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer, PerkinElmer, Inc.).

Determination of Newly Synthesized TGs in Mammary Cells HepG2 cells (5 $\times 10^5$ cells/mL) were incubated with dimethyl sulfoxide (DMSO) or selected DGAT2 inhibitors (H1-001 to -008) at 10 μ M in the presence of [¹⁴C]-glycerol (0.6 μ Ci) for 6 h. At the end of the incubation, intracellular lipids were extracted with a mixture of hexane–isopropanol (3:2, v/v) and separated on a TLC plate using a hexane–diethyl ether–acetic acid (80:20:1, v/v/v) solution as a developing solvent. The isotope-labeled TGs were detected and quantified with a bio-imaging analyzer (FLA-7000, FUJIFILM).

Plasmid Construction and Establishment of Stable HEK293 Tet-On Cell Lines The pTRE2-hygro (Clontech) was used to tightly regulate the expression of human DGAT1 and DGAT2. Each cDNA was amplified by PCR and inserted into the pTRE2-hygro vector. Stable HEK293 Tet-on cells expressing DGAT1 or DGAT2 were established by transfection of pTRE2-hygro-DGAT1 or pTRE2-hygro-DGAT2, followed by selection with hygromycin for 2 weeks. Clonal cell lines were tested for the expression of DGAT1 or DGAT2 in the presence and absence of doxycycline by Western blot analysis and staining with a Bodipy 493/503 dye (Molecular Probes).

Analysis of Lipid Droplet Formation in 3T3-L1 Cells In order to examine whether compounds effectively inhibited DGAT2 activity, preadipocyte 3T3-L1 cells were plated in a 24-well plate at a density of 0.5 $\times 10^4$ cells/well. After 24 h, cells were treated with compounds in the presence of 100 μ M oleic acid. After incubation for two days, cells were fixed with 2% formaldehyde in phosphate-buffered saline for 20 min and stained with a Bodipy 493/503 dye (Molecular Probes) and Hoechst 33342 (Sigma-Aldrich). Lipid droplets were analyzed using the Cellomics ArrayScan instrument (Cellomics Inc.) with version 3.1 equipment and software module, known as the Spot Detector BioApplication.

Statistical Analysis All results are presented as the mean $\pm$ standard deviation (S.D.) of independent experiments ($n \geq 2$).

RESULTS

Identification of a Novel Class of DGAT2 Inhibitors by High-Throughput Screening In our previous study, we performed a high-throughput screen of 20000 compounds derived from the ChemBridge chemical library and identified a few compounds, including compound 122, that consistently showed DGAT2 inhibitory activity.¹⁷ In this process, we found out a class of compounds that commonly have a 1*H*-pyrrolo[2,3-*b*]pyridine core structure; these molecules were designated as H2-series (Fig. 1A). In the following assay, their DGAT2-inhibitory activities were further confirmed by using a conventional extraction-based *in vitro* DGAT2 assay, in which the newly synthesized isotope-labeled TG was extracted by a series of combinations of organic solvents, and then the radioactivity was quantified. As a result, all eight compounds exhibited a potent inhibition of DGAT2 activity, with IC₅₀ values of 5–30 μ M (Fig. 1B). Given that they all possess a 1*H*-pyrrolo[2,3-*b*]pyridine core structure, it seems that this shared moiety contributes primarily to their inhibitory activity while other arms confer differential activities to each compound.

Fig. 1. Identification of a Novel Class of DGAT2 Inhibitors with a 1*H*-Pyrrolo[2,3-*b*]pyridine Core Structure

(A) Structures of eight compounds (designated H2-series) that possess 1*H*-pyrrolo[2,3-*b*]pyridine as a core structure (dashed rectangle) (B) The IC₅₀ values of H2 series compounds in (A) on DGAT2 and its isozyme DGAT1 were determined using a conventional extraction-based *in vitro* DGAT assay, as described in 'Determination of *in Vitro* DGAT Enzymatic Activity' in Materials and Methods.

Fig. 2. Suppression of TG Synthesis in HepG2 Cells by H2-Series Compounds

(A) HepG2 cells were treated with 10 μM of H2-series compounds in the presence of [¹⁴C]-glycerol for 6h, and then a TLC-based TG assay was performed with lipid extracts from the cells (see 'Determination of Newly Synthesized TGs in Mammalian Cells' in Materials and Methods). Each TG band was quantified using Multi-Gauge V3.0 (FUJIFILM). Cell viability was measured using CellTiter Glo reagent after treatment with 10 μM of compound for 24h. The relative activities were calculated by setting the values from DMSO-treated cells to 100%. (B) HepG2 cells were treated with 10 μM of compound for 48h, and then LD spots were measured by staining with a fluorescent Bodipy dye and quantifying the number of LD spots using Cellomics BioApplication analysis software. The relative number of LD spots was calculated by setting the values from DMSO-treated cells to 100%.

Next, in order to determine the selectivity of these compounds, we examined their effect on the activity of DGAT1 using the total membrane fraction of human DGAT1-overexpressing Sf-9 cells. DGAT1 is the other isozyme of DGAT2. Both enzymes share similar specific activity with respect to substrates for TG synthesis. As shown in Fig. 1B, most of the compounds had little or weak inhibitory effect on DGAT1, while showing a marked effect on DGAT2. Overall IC₅₀ values of all eight compounds against DGAT2 were about 10-fold lower than those against DGAT1. Collectively, these results indicate that a novel class of compounds with a 1*H*-pyrrolo[2,3-*b*]pyridine core potentially and preferentially inhibits the activity of DGAT2 *in vitro*.

H2-Series Compounds Inhibit TG Biosynthesis in Hepatic Cells To determine whether H2-series compounds also suppress TG biosynthesis at the cellular level, we first examined the effect of these molecules on newly synthesized TG in

HepG2 hepatic cells. Previous studies suggested that DGAT1 and DGAT2 mediate distinct hepatic functions despite catalyzing the same biochemical reaction.¹⁵ DGAT2 preferentially esterifies exogenously added glycerol to endogenously synthesized fatty acids, while DGAT1 preferentially esterifies exogenously added fatty acid to already existing diacylglycerol. Therefore, we measured the newly synthesized isotope-labeled TG in HepG2 cells by adding [¹⁴C]-labeled glycerol as an exogenous substrate of DGAT2. As a result, treatment of all eight compounds at 10 μM considerably decreased incorporation of [¹⁴C]-glycerol into TG in HepG2 cells to different extent (Fig. 2A; H2-002, -003, -004, -005, and -008 showed 10–20% inhibition; H2-001, -006, and -007 exhibited 30–45% inhibition of TG formation). In contrast, the same treatment had little effect on the viability of HepG2 cells, which was determined using the CellTiter Glo reagent, which measures ATP concentration.

Hepatic cells generally accumulate surplus energy as lipid

Fig. 3. Selective Inhibition of DGAT2 by H2-003 and -005 in DGAT2- or DGAT1-Overexpressing HEK293 Cells

(A) HEK293 cells inducibly overexpressing DGAT1 or DGAT2 were treated with doxycycline for 24h, stained with a Bodipy dye, and visualized by inverted fluorescence microscopy. (B) DGAT activities were measured with total membrane proteins from DGAT2- or DGAT1-overexpressing cells at various concentrations of compounds H2-003, -005, or DGAT1 inhibitor [*N*-(6-morpholinopyridin-3-yl)-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide], and then the IC₅₀ for each was determined.

droplets (LDs) in the cytoplasm that are majorly composed of TG.¹⁸⁾ In order to assess whether H2-series compounds also have an effect on steady-state TG level, we measured LD spots by staining with a fluorescent Bodipy dye and quantifying the number of LD spots using Cellomics BioApplication analysis software (Fig. 2B). As a result, four out of eight compounds (H2-001, -003, -005, and -007) showed a considerable decrease in LD formation. In particular, H2-003 and -005 exhibited the strongest inhibition with nearly 50% reduction in LD formation. Therefore, we conducted the remaining experiments in this study using only these two compounds. Discrepancy in data from two different experiments (Figs. 2A, B) could be due to different stability of each compounds, since newly synthesized TG and steady-state TG were measured at different times (6, 48h, respectively) after compound treatment. Taken together, these data demonstrated that H2-003 and -005 inhibit the DGAT2 activity in HepG2 cells, resulting in suppression of TG formation.

H2-003 and -005 Selectively Inhibit DGAT2 Activity in Mammalian Cells Mammalian cells generally express

both DGAT isozymes with the proportion of each protein varying depending on the cell type.^{15,19)} Therefore, it is not easy to assess the selective action of a compound on an individual DGAT at the cellular level. In order to overcome this challenge and examine whether H2-003 and -005 selectively inhibit DGAT2 in mammalian cells, we first established HEK293 cell systems that inducibly overexpress DGAT2 or DGAT1 upon the addition of doxycycline. As shown in our previous report,¹⁷⁾ overexpression of either DGAT2 or DGAT1 alone drastically increased the fluorescence signal of LD by more than 20-fold compared with control cells that showed a faint basal signal (Fig. 3A).

Next, we examined the selective inhibitory activity of H2-003 and -005 by measuring DGAT enzymatic activities using total membranes isolated from DGAT2- or DGAT1-overexpressing cells as enzyme sources. Compounds H2-003 and -005 showed a strong inhibitory activity on DGAT2 with IC₅₀ values of 7.1 and 6.1 μM, respectively, while showing weak inhibitory activity on DGAT1 with a much higher IC₅₀ value of >100 μM (Fig. 3B). Conversely, a reference DGAT1-selective inhibitor strongly affected DGAT1 activity with an IC₅₀ value of 0.7 μM, but exhibited a weak effect on DGAT2 activity (Fig. 3B). Moreover, we examined the selective effect of compound H2-003 on DGAT2 by measuring the altered number of LDs in both cells. The number of LDs formed by dose-dependent treatment of compound H2-003 was significantly reduced, with IC₅₀ of 5 μM, in DGAT2-overexpressing cells (Supplementary Fig. 1A), but to a lesser extent in DGAT1-overexpressing cells (Supplementary Fig. 1B). Similar results were observed with compound H2-005 (data not shown). At the same condition, DGAT1 inhibitor remarkably decreased LDs only in DGAT1-overexpressing cells. Taken together, our data demonstrate that compounds H2-003 and -005 significantly suppressed TG formation induced by overexpression of DGAT2, but not DGAT1, indicating that H2-003 and -005 exert a selective inhibitory activity on DGAT2 in mammalian cells as well.

Combined Treatment of H2-003 or -005 with a DGAT1 Inhibitor Effectively Suppresses LD Formation in Preadipose Cells A previous study showed that both DGAT1 and DGAT2 are required for LD formation in adipocytes. LD formation was severely affected by double knockout of these enzymes, but was weakly impaired by single knockout of ei-

Fig. 4. Combined Treatment of H2-003 or -005 with a DGAT1-Selective Inhibitor Effectively Suppresses LD Formation in 3T3-L1 Cells

3T3-L1 cells were treated with various concentrations (1, 3, 5, and 10 μM) of (A) H2-003 or (B) -005 with or without 2 μM DGAT1 inhibitor [*N*-(6-morpholinopyridin-3-yl)-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxamide] in the presence of 100 μM oleic acid for 48h. LD spots were then quantified after staining with a Bodipy dye. The relative number of LD spots was calculated by setting the values from DMSO-treated cells to 100%. The *p*-values were depicted by asterisk (**p*<0.01, ***p*<0.001).

ther protein.¹³) To confirm this observation pharmacologically, we examined the combined effect of H2-003 or -005 with DGAT1 inhibitor on LD formation induced by the treatment of oleate in 3T3-L1 cells. Oleate-induced 3T3-L1 cells were treated with increasing concentrations of each DGAT2 inhibitor alone or with a fixed concentration of a DGAT1 inhibitor for 48h. The optimal concentration of the DGAT1 inhibitor was determined by preliminary titration to be 2 μ M, which corresponds to suppression of LD formation by about 50%. As shown in Fig. 4, each DGAT2 inhibitor by itself suppressed LD formation, particularly at 10 μ M, which was the highest concentration. Furthermore, co-treatment of H2-003 or -005 with the DGAT1 inhibitor more effectively suppressed LD formation (Fig. 4). In particular, combined treatment of 10 μ M H2-003 or -005 with the DGAT1 inhibitor almost completely reduced LD formation to as low as the basal level in DMSO-treated cells, which was not attained by either DGAT1 or DGAT2 inhibitor alone. Thus, we validated the functionality of H2-003 and -005 pharmacologically as DGAT2-selective inhibitors in the context of DGAT1 inhibition.

DISCUSSION

TG synthesis plays a significant role in the pathogenesis of metabolic diseases such as obesity, hepatic steatosis, hyperlipidemia, and insulin resistance.^{2–4,20} Recently, several enzymes in the TG biosynthetic pathway, including DGAT, ACAT, GPAT, and FAS, have emerged as a new class of therapeutic target for the treatment of metabolic diseases. Of these enzymes, DGAT1 and DGAT2 are of particular interest since they catalyze the terminal and only committed step in TG synthesis. Initially, DGAT1 drew major attention because of its therapeutic potential, which was suggested by weight loss and improved glycemic control with a moderate reduction of TG in *DGAT1* knockout mice. In contrast, DGAT2, in spite of its profound contribution to TG biosynthesis in the mouse, has been somewhat neglected because of potential risk indicated by postnatal death in DGAT2-deficient mice due to TG deficiency and abnormality in the skin.⁷) More recently, a series of experiments using adult rodents with reduced levels of DGAT2 protein have shown that targeting this enzyme would be beneficial to treating hepatic steatosis, hyperlipidemia, and even type II diabetes.^{10,21,22}) However, pharmacological validation of DGAT2 *in vivo* remains to be investigated.

In another aspect, even though DGAT1 and DGAT2 take part in the same reaction of the TG biosynthetic pathway, their biochemical properties seem to be somewhat different. In order to define the distinct role of each protein, it is crucial that DGAT2 is differentiated from DGAT1 by using a selective pharmacological inhibitor. Therefore, a selective inhibitor of DGAT2 is needed not only to evaluate its therapeutic potential but also to provide a tool for pharmacological study of the role of DGAT2 in TG synthesis.

Here, we discovered eight compounds sharing a 1*H*-pyrrolo[2,3-*b*]pyridine core structure as a novel class of DGAT2 inhibitors through extensive high-throughput screening, and confirmed their effectiveness and selectivity in various assays involving mammalian cells and a cell-free system. Given that all the compounds possess a 1*H*-pyrrolo[2,3-*b*]pyridine core structure, it seems that this common moiety contributes primarily to their inhibitory activity while other

arms confer differential activities to each compound. Intriguingly, DGAT2 inhibitors were recently reported as indolyl acrylamide derivatives, which are structurally similar to the azaindolyl core structure of our H2-series.¹⁶) According to this study, protecting the nitrogen within the indole ring is important for their DGAT2 inhibitory activity. In that sense, it is noteworthy that the nitrogen of the azaindole ring in all our H2-series compounds is protected (Fig. 1A). Future structure–activity relationship studies will define the critical part required for full activity and ultimately improve pharmacological properties such as efficacy, which is required for *in vivo* validation. Of the eight compounds, H2-003 and -005 consistently showed a considerable inhibitory effect on TG synthesis in hepatic cells (Fig. 2), as well as cell-free DGAT2 activity (Fig. 1). Therefore, we focused on these two compounds for further analysis.

One of the most important requirements for a DGAT2 inhibitor is its selective action on DGAT2 over other enzymes involved in lipid metabolism, particularly DGAT1 isozyme. High selectivity of H2-003 and -005 to DGAT2 was demonstrated using many experimental settings. First, compounds H2-003 and -005 strongly inhibit DGAT2 with only a marginal effect on DGAT1 in cell-free enzymatic assays (Fig. 1B). Second, H2-003 and -005 affected steady-state TG formation as well as the incorporation of [¹⁴C]-glycerol into TG in HepG2 cells (Fig. 2A). This data is supported by a recently report showing that DGAT2 is mainly responsible for TG synthesis involving endogenously originated fatty acids.¹⁵) Third, H2-003 and -005 exhibited more potent DGAT inhibitory activity in DGAT2-enriched membrane fractions than DGAT1-enriched ones prepared from DGAT2- or DGAT1-overexpressing HEK293 cells. Finally, combined treatment of H2-003 or -005 with a DGAT1 inhibitor effectively suppressed oleate-induced LD formation in 3T3-L1 preadipose cells. Either DGAT1 or DGAT2 inhibitor alone did not attain this effect. Collectively, we clearly demonstrated that H2-003 and -005 are a novel class of DGAT2-selective inhibitors. Nevertheless, we can not completely exclude the possibility that H2-003 and -005 may affect other untested enzymes involved in lipid metabolism that have acyl-CoA as their substrate.

In conclusion, we identified two novel compounds, H2-003 and -005, as potent and selective inhibitors of DGAT2. These compounds would be useful tools for the pharmacological study of DGAT2-related biology. Further validation in disease models is needed to determine other therapeutic options for treating several metabolic diseases.

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